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Army Medical Research Unit
Kuala Lumpur, Malaysia

1 July 1972

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UNITED STATES ARMY MEDICAL RESEARCH UNIT

INSTITUTE FOR MEDICAL RESEARCH

KUALA LUMPUR, MALAYSIA.

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13. ABSTRACT

INVESTIGATIONS OF BACTERIAL DISEASES

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Comparisons of the Gram stained smear with the results of quantitative stool culture, made by six observers, suggest that the method in its present form is of doubtful use as a screening aid for the clinician. However, there was a tendency for the observers to be in agreement, whether or not their findings equated to the culture results. This suggests that further work in a different setting may lead to the successful development of a screening test.

An outbreak of pertussis-like illness is described. The etiology suggested is that of an Adenovirus infection.

ECOLOGICAL STUDIES OF MAMMALS AND THEIR INVOLVEMENT IN TRANSMISSION OF ZOONOTIC DISEASES IN EQUATORIAL ECOSYSTEMS

Data from field studies are beginning to support the conceptual role of medical ecology (Muul, 1970, Science, 170: 1275-1279; Abu Bakar bin Ibrahim, Muul, and Lim, 1971. Mimeographed. Institute for Medical Research). Understanding the enzootic transmission cycles of zoonotic pathogens in natural hosts may lend predictive value

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KEY WORDS	ROLE	WT	ROLE	WT	ROLE	WT
Bacteriology, diarrhea, pathogen excretion pattern, *Shigella, Salmonella, E. coli,* Gram stain, fecal smears, early diagnosis of diarrhea, Malaysia, *Bordetella pertussis,* Adenovirus, pertussis syndrome, *Orang Asli,* Aborigine, Medical Ecology, ecological niches, vertical zonation, species diversity, zoonotic pathogens, *Rickettsia tsutsugamshi,* habitats, tropical rainforest, seasonal phenomena, serological results, canopy transect walkways, blood parasites, trapping, West and East Malaysia, enzootic transmission, forest canopy, arboreal mammals, silvered leaf-monkeys, *Presbytis cristatus, Rattus annandalei,* mouse deer, *Tragulus javanicus,* laboratory animal medicine, liver trauma, liver regeneration, malaria, *in vitro, Anopheles,* canopy mosquitoes, chloroquine resistance, culicine, malaria survey, Plasmodium, Tragulid malaria, West Malaysia, malaria, liver stage of malaria, drug resistance, *in vitro,* experimental infection, parasitology, rickettsia, scrub typhus, leptotrombidium, *R. tsutsugamushi,* tick typhus, ixodid ticks, rickettsia, hemolymph screening.						

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U.S. ARMY MEDICAL RESEARCH UNIT
INSTITUTE FOR MEDICAL RESEARCH
Kuala Lumpur, Malaysia

ANNUAL RESEARCH PROGRESS REPORT
1 July 1971 - 30 June 1972

US ARMY MEDICAL RESEARCH
AND DEVELOPMENT TECHNICAL REPORT

1 July 1971 - 30 June 1972

SUMMARY

INVESTIGATIONS OF BACTERIAL DISEASES

Methods previously described were used to evaluate the Gram stained fecal smear as a rapid diagnostic tool in diarrheal disease.

Comparisons of the Gram stained smear with the results of quantitative stool culture, made by six observers, suggest that the method in its present form is of doubtful use as a screening aid for the clinician. However, there was a tendency for the observers to be in agreement, whether or not their findings equated to the culture results. This suggests that further work in a different setting may lead to the successful development of a screening test.

An outbreak of pertussis-like illness is described. The etiology suggested is that of an Adenovirus infection.

ECOLOGICAL STUDIES OF MAMMALS AND THEIR INVOLVEMENT IN TRANSMISSION OF ZOONOTIC DISEASES IN EQUATORIAL ECOSYSTEMS

Data from field studies are beginning to support the conceptual role of medical ecology (Muul, 1970, Science, 170: 1275-1279; Abu Bakar bin Ibrahim, Muul, and Lim, 1971. Mimeographed. Institute for Medical Research). Understanding the enzootic transmission cycles of zoonotic pathogens in natural hosts may lend predictive value to anticipating the epidemics in unnatural hosts, such as man, under specific circumstances. For example, the habitat of greatest enzootic activity of scrub typhus (Rickettsia tsutsugamushi) in Malaysia is the forest rather than the classical "scrub" habitat previously described. This was found to be the case for areas tested both in West and East Malaysia (Sabah). In both areas arboreal rodents did not appear to be involved, not even as the postulated nidus or primordial source for infection (Audy, 1961, in May, J.M. ed., Studies in Disease Ecology, Hafner, N.Y.). Seasonal effects appear to be operant, although it is not possible to determine at this time whether it is a matter of reinfections or synchronous recrudescence of latent infections. Seasonal effects may obscure the results of short-term surveys. Species seem to respond differentially to infections with zoonotic pathogens as predisposed by their ecological niches. In surveys, trap response has to be considered. In survey trapping, many species of arboreal mammals are missed. These are actually shown to be abundant when other collecting methods (such as capture by hand from arboreal tree cavities) are employed.

Studies with the aid of the canopy transect walkway system (Muul & Lim, 1970, Science, 169: 788-789) have shown that there is little overlap in species diversity in the forest canopy and that on the ground. Rates of parasitization, such as with *Plasmodium* differ also according to vertical zonations. Forests that appear similar differ markedly in their species diversity of mammals depending on the age and history of the forests. Prevalences of various blood parasites also differ in various habitats (e.g. Muul, Lim, and Yap, 1970. S.E. Asian J. Trop. Med. & Publ. Hlth., 1(3): 418-419). Additional data for seasonality studies of ecological phenomena and temporal distribution of zoonotic pathogens are being accumulated and analyzed.

LABORATORY ANIMAL DEVELOPMENT AND ZOONOTIC DISEASES

No major changes were made in the management of the breeding colonies during the last year except for the guinea pigs where new breeding stock was obtained from WRAIR. The rat and hamster colonies were recaged and brought up to standard. A new laboratory animal facility was designed for the IMR in cooperation with the Department of Veterinary Medicine, SEATO Medical Research Laboratory. This will be a modern facility and will bring the laboratory animal facilities at the IMR up to modern standards. Construction is to start 1 November 1972 with a completion date of December 1973.

Procedures were standardized for the capture, handling and conditioning of silvered leaf-monkeys. Current methods are yielding a survival rate of 67%. No losses have been encountered in silvered leaf-monkeys after the first two months in the laboratory and one group has now been in the laboratory over 1 year. In order to cut losses and to obtain a 67% survival rate, it was necessary to take charge of the animals at the time of their capture and immediately bring them to the laboratory. Exhaustion and shock are the largest killers of the animals, most deaths occurring within 5 days of capture.

Normal values for silvered leaf-monkeys for rectal temperature, PCV, RBC, WBC including differential and serum protein have been determined. It was found that the animals suffer from a normocytic, normochromic anemia in the wild state which corrects itself in the laboratory over an eight week period. Serological work up of monkeys revealed that in contrast to pig-tailed and cynomolgus monkeys the silvered leaf-monkey does not carry antibodies to six of the endemic diseases of Southeast Asia.

Mouse deer survival and breeding continued to improve. The survival rate is now 70% on wild caught animals that arrive at the laboratory within 24 hours of capture. Several offspring were born during the year. The *Rattus annandalei* colony continued to expand and production records revealed a litter size of 4.3 (range 1-7) with a mean interval of 41.5 days between litters.

INVESTIGATIONS ON LIVER *IN VITRO*

Investigations on Normal Liver Tissue *In vitro*

Attempts were made to maintain explants of normal liver *in vitro* in order to study factors which promote liver regeneration and also to attempt to grow the primary exoerythrocytic stage of malarial parasites for possible studies of growth requirements of and drug action on this important stage.

Liver from pig-tailed macaques *(Macaca nemestrina)* was collected at surgery, cut into $1\frac{1}{2}$ mm cubes and placed in collagen lined tubes containing tissue culture medium M 199 and homologous serum and maintained at 39°C. Medium and serum were changed every third day. Biochemical monitors of liver parenchymal functions - production and storage of glycogen, production and excretion of cholesterol and albumin - were adopted in order to evaluate stimulation or inhibition of the liver. At various times of incubation C-14 labelled glucose or sodium acetate was added to the medium and determination was made of the amount of isotope incorporated after 2 days into the tissue glycogen, excreted cholesterol, and albumin. Technical problems were encountered in histological preparation possibly due to the friability of the infarcted cores of the liver pieces.

When isotope labelled substrates were added to the incubation mixtures of explanted liver, M 199 and serum the label was reproducibly incorporated into the three fractions. The times of incubation prior to pulsing with isotope which have been tested thus far are 1 day, 4 days, 8 days, 12 days, 16 days, 20 days, 24 days, 28 days. Longer studies are in progress. Serum collected after partial hepatectomy appeared to promote greater incorporation of isotopes into the three fraction than normal, pre-hepatectomy serum. In general serum collected at 2 and 3 weeks following surgery seemed to stimulate incorporation of label into all fractions. Heat "inactivation" of both pre and post hepatectomy sera at 56°C for 30 minutes caused a reduction of isotope incorporation into the three fractions. In preliminary experiments, incorporation of label appeared comparable whether or not collagen was used in the tubes and whether continuous rolling or static conditions were used. Continuous rolling, however, was the routine method for incubation.

Because fairly high background-counts in all three fractions were encountered when heat killed liver was used, and in the chloroform extract and TCA precipitate fractions when no liver tissue was added, further work is under way to confirm and clarify the earlier findings.

INVESTIGATIONS OF MALARIA

Basic investigations on chloroquine resistant *Plasmodium falciparum* and associated mosquito vectors were continued during this reporting period. Studies of malaria in the *Orang Asli* (Aborigines), and the effect of weekly prophylaxis and residual insecticide spraying (DDT) every three months on their malaria rate, was evaluated. Supervision by Gombak Aborigine Hospital staff appears to be the key to the success of the program.

Ecological and entomological research on the mosquito fauna at ground and canopy level have been concluded.

Intensive studies of tragulid (mouse deer) malaria have been carried out. A new species of *Plasmodium* almost three times the size of *P. traguli* has been found. The sporogonic stages of *P. traguli* were studied by light microscopy and the erythrocytic stages by electron microscopy. Transmission experiments were also carried out, with the new species apparently being transmitted by *Anopheles* mosquitoes. Studies of *P. youngi* were initiated. Possibilities exist that the strain is naturally resistant to chloroquine although a secondary exoerythrocytic cycle could be involved. A colony of *A. letifer* has been established and maintained to support the transmission experiments with *P. traguli*, and the new *Plasmodium* species.

Investigations of Malaria *In vitro*

Investigations on the Exoerythrocytic Stages of Malaria *In vitro*

In association with the normal liver tissue culture work attempts were made to reproduce *in vitro* the primary exoerythrocytic stage of *Plasmodium cynomolgi*; as a model for this stage for studies of growth requirements and drug testing.

Salivary glands containing sporozoites of *Plasmodium cynomolgi* were dissected from experimentally infected *Anopheles maculatus*, and added to the liver explants after various periods of culture. At 8, 10, and 12 or 14 days after addition of the sporozoites, liver tissue was inoculated intraperitoneally into a splenectomized pig-tailed macaque *(Macaca nemestrina)*. Simultaneously some of the liver tissues were prepared for histological examination. Medium from the cultures was inoculated into a control splenectomized monkey. All monkeys were kept in screened quarters to avoid accidental mosquito borne infection.

Histological preparations of the cultured liver were unsatisfactory, possibly due to the friability of the infarcted cores of the liver.

In the first of the four completed experiments the medium recipient monkey remained negative for patent malarial infection. However, the tissue recipient became positive. The first parasites appeared 32 days after splenectomy, 20 days after the first tissue inoculation, but only 6 days after the last inoculation. This monkey had the appropriate *Plasmodium cynomolgi* with a pattern of parasitemia consistent with a primary infection. This monkey had been in the laboratory for more than four years prior to this work.

In the second experiment, the tissue recipient was found to have only three parasites, which appeared 9 days after the last inoculation and 18 days after first tissue inoculation. This was greater than 2 months post splenectomy. While the three parasites were clearly asexual malarial parasites, the species could not be identified with certainty. The medium recipient monkey remained negative.

The subsequent two experiments, which had slight modification of the liver culture technique, did not result in detectable parasitemia.

Further attempts are under way to reproduce the first findings. Further modifications of the histological techniques are being attempted.

Investigations on Drug Resistance of *Plasmodium falciparum In vitro*

Attempts are being made to modify the *in vitro* method of Diggs *et al* (J. Parasit.: 57, 187-188, 1971) for field studies in Malaysia. This work is in the preliminary stages. It is anticipated that this method, if feasible, and the Rieckmann method already in use will be employed in the field to determine what correlation might exist between these *in vitro* systems and the parasitological and clinical effect of the drug in the patient.

INVESTIGATIONS OF SCRUB TYPHUS

Microdissection and the direct fluorescent antibody technique were used to demonstrate scrub typhus rickettsia in all stages of infectious *Leptotrombidium (L.) fletcheri (=akamushi)* from the positive colony. The gut tissues and hemolymph were positive in all post egg stages. Unengorged larvae had the highest percentage of tissues positive for rickettsia. Of eggs taken from known infectious females, 91.7% were positive by FA examination. Examination of egg contents with the FA technique appears to be a feasible means for screening field collected vectors for colonization, since the adult is kept alive.

The infected *L. (L.) fletcheri* colony is into the 11th laboratory generation. Observations of sex ratios in the infectious and non-infectious colonies of this species suggest that a type of parthenogenesis, possibly thelyotokus gynogenesis, is responsible for the lack of males in the positive colony.

L. (L.) deliense numbers are being sampled in a variety of adjacent habitats by black plate and rodent collections. Comparisons are incomplete at present, but monthly fluctuations in numbers do not consistently correspond with average monthly rainfall alone. During some months, marked differences occurred in the average numbers of chiggers on rodents and those on black plates. The number of chiggers on male *Rattus argentiventer* and *R. tiomanicus jalorensis* were notably higher than those on females of the same species. *R. argentiventer* was the most important and *R. exulans* the least important host species in the study area in terms of numbers of chiggers per infested rat.

It was shown in five experiments that the larvae of the vector Leptotrombidium mites *(L. fletcheri, L. deliense, L. arenicola)* can take up rickettsial organisms from infected rodents (mice and rats). However, to date no transovarial transmission has been demonstrated. The efficiency with which vector mites take up rickettsial organisms

from infected rodents appears to be species dependent. Unless
transovarial transmission occurs in experime s which are not yet
completed, it is reasonable to suspect that t e vector mites do not
become infected from rodents but are themsel s both the reservoir
and vector of scrub typhus.

Silvered leaf-monkeys seem to be an cellent subhuman primate
model for human scrub typhus and all res nses measured were strain
and dose dependent. Three strains fa ed to produce eschars at any
dose (up to 10^6) while others produce eschars with doses as low as
$10^{1.5}$. Significant titers were obtained to the minor antigenic
components of the strains in addition to the major components.
Complete protection, as determined by clinical illness, was demonstrated
in silvered leaf-monkeys challenged at six months with homologous,
homologous-heterologous or heterologous combinations of strains.
Immunity affected both the formation and duration of eschar formation.

The organism was shown to be antigenically stable in silvered
leaf-monkeys, vector mites and a wild rodent. The minor Karp
component of Kato varies in its degree of expression both in vector
mites and silvered leaf-monkeys.

Storage studies of high titer material showed that within the
limits tested freezing and thawing rates had little or no effect on
the titers obtained and that through six months storage that there was
no significant differences between the titers of the material stored
at $-65^{\circ}C$ and $-175^{\circ}C$. Materials stored at both temperature lost $1 \log_{10}$
of titer in six months.

An area was selected for a long term study of the effect of
season and habitat on mammalian isolation and serology ratios.
Indicator species for each habitat were selected and the study
initiated for the following habitats: a village area, edge habitat,
lalang grass and relict primary/secondary forest.

INVESTIGATIONS OF TICK TYPHUS

A total of 105 Ixodid ticks of 6 species were collected from an
area where tick typhus had previously occurred and were screened for
rickettsial infection by examination of hemolymph slides. Examination
of both giemsa and indirect fluorescent antibody preparations indicated
that 2 *Haemaphysalis papuana* and *watremi* were infectious with a Rocky
Mountain Spotted fever-like rickettsia. *Rickettsia conorii*-like
infections were indicated by hemolymph screening in 2 *H. p. * *indiatrami*
and 1 *H. semermis*. These ticks are being colonized for further study.

FOREWORD

In conducting the research described in this report, the
investigator(s) adhered to the "Principles of Laboratory
Animal Care as established by the National Society for
Medical Research."

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23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) Technical Objectives: (1) To relate Gram stain appearance of fecal smears to patterns of pathogen excretion in normal and diarrheal populations by methods simple enough for use as screening in outpatients departments. (2) To attempt definition of the causative agent in a pertussis-like epidemic.

24.(U) Approach: (1) Fresh stool specimens diluted, mixed and examined by staining of fecal smear. Photomicrography of stained smears and comparison with results of quantitative and qualitative stool culture.

25.(U) Progress: (1) Further patterns of pathogen excretion were demonstrated in early diarrhea, showing balance between host and bacterial parasite.

(2) 511 photomicrographs representing 60 human and 3 monkey sequential stool collections assessed by six observers independently. 12.5 per cent false negative assessments render the method, with present criteria, unsuitable as a screening technique.

(3) No isolates of *Bord. pertussis* from 38 children during a pertussis-like epidemic. One death from interstitial, viral pneumonia occurred; rise C.F. titers to adenovirus was noted in 3 patients. Adenovirus is proposed as the cause.

Keywords: Bacteriology, diarrhea, pathogen excretion pattern, *Shigella, Salmonella, E. coli,* Gram stain, fecal smears, early diagnosis of diarrhea, Malaysia, *Bordetella pertus* , Adenovirus, pertussis syndrome, *Orang Asli,* Aborigine.

* Pediatrician, University Hospital, Kuala Lumpur, Malaysia.

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INVESTIGATIONS OF BACTERIAL DISEASES

The effort of the present reporting year has been concerned in attempting to consolidate the concepts of the previous two years and, more specifically, has been aimed at determining if a relationship exists between the appearances of the Gram stained fecal smear and numbers of pathogen isolated from stool cultures.

Medical care for unit personnel has continued to consume several hours each week with a broad spectrum of complaint presented. The bulk has been viral upper respiratory disease; some have consisted of episodes of dengue and assorted conditions such as chloroquine resistant malaria acquired a short distance from Kuala Lumpur; inguinal hernia; a possible carcinoma of stomach presently under investigation and several instances of infected leech bite. There has been no serious illness among the US Army personnel or their dependents.

The laboratory at Gombak Aborigine Hospital remains part of unit responsibility and has contributed two items of interest during the year, namely, an episode of pertussis-like illness and several cases of cholera due to *Vibrio cholera* El tor, Ogawa. In each instance information of the outbreaks was slow in reaching USAMRU.

The department of bacterial diseases continues to support the diagnostic and investigative needs of the rest of the unit in terms of preparation of media, investigation of specimens from the animal colony and of human material and also in antibiotic sensitivity testing. It also continues to be involved in aspects outside of the immediate unit reference, such as quality control of the water supply for part of the National Zoo, assistance with bacteriological investigations in the General Hospital, Kuala Lumpur and, on an occasional basis, in giving hematologic opinion for one general medical practitioner.

A proposal to commence collaborative work on *Yersinia enterocolitica* with the University of Malaya Department of Surgery has not yet matured due to pressure of work at that department.

Within the last month work on disease transmission by the leech, which was the subject of an observation in the Annual Report of 1967, has been re-opened.

Investigations of Diarrhea

During a mild outbreak of cholera in the autumn of 1971, the local population was advised by the Ministry of Health to attend General Hospital at the first sign of diarrheal disease. Because of this, several patients were detected very early in the course of bacterial diarrheal disease. Using methods described in previous annual reports further demonstration of variations in the numbers of pathogens excreted by non-treated patients was provided. Figure 1 shows these
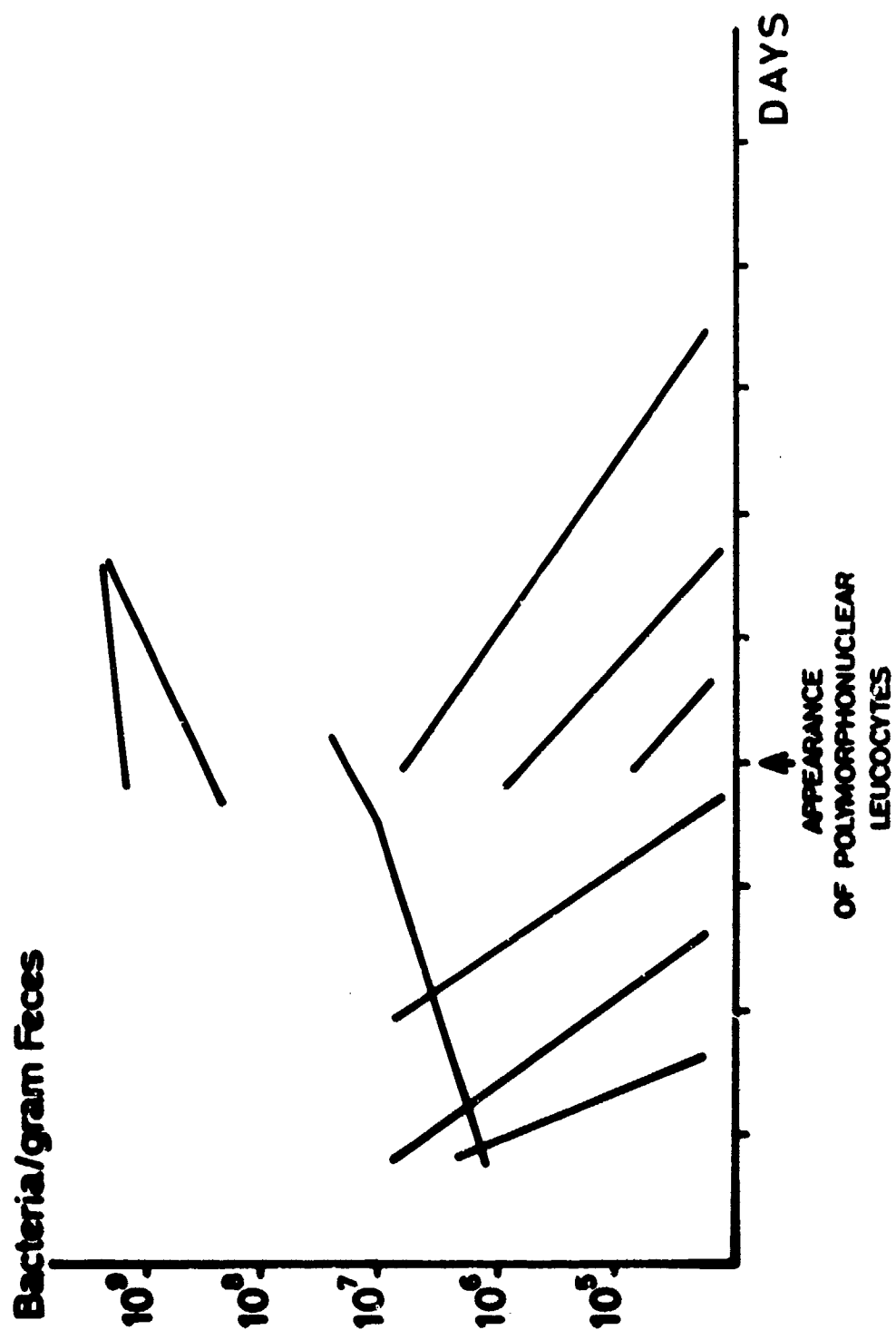
FIG. 1   EXCRETION PATTERNS OF PATHOGENS IN QUANTITATIVE STOOL CULTURE IN EARLY DIARRHEA

excretion curves indicating that excretion changes with time, the kinetics differing from patient to patient. The slopes of the curves presumably reflect host-parasite relationship, giving some idea of the "balance of power". In the figure there is an apparent discontinuity in the families of increasing and decreasing slopes, the discontinuity being found, not at 10^8 but below 10^5, in the carrier state. It is noticeable also from Figure 1 that the presence of polymorphonuclear leucocytes in a stool sample is not a reliable predictor of the progress of the disease.

The changes which occur in the aerobic bacterial flora of feces in patients with diarrhea were the subject of a logical approach which has been defined in the previous two annual reports. These changes were expressed by the results of continued reproduceable quantitative culture and by the observation of shifts in population size on Gram staining of stool suspension, but results so far had been obtained from small numbers of patients. The bulk of this year's work was directed at studying two parameters only - the Gram smear and quantitative culture - in an attempt to show whether the first mirrored the second, with a view to using the Gram smear as a rapid outpatient technique in giving the physician a quick, reliable screening tool. To be of any use in this respect only easily detected alterations in the populations of the smear should be considered, since the method would be most often applied in areas where partly trained technicians are available. In this respect it differs from the previous method, where accurate, time-consuming measurements were made. However, the methods of stool collection and preparation remain as previously reported.

Three study sites were set up, representing different types of age and medical status populations. Extra technical help was recruited in the form of eight medical students from the University of Malaya, each of whom had just passed the professional examinations in pathology, parasitology and bacteriology.

Four students were stationed in Kuala Brang, a town in the State of Trengganu, some 290 miles by road, north east of Kuala Lumpur. They were housed and worked in a vacant building of the local medical center. Conditions there were not ideal, there being no piped water to the building, no air-conditioning and little refrigeration facilities. Supply of materials and return of plated stool and Gram smears was arranged on a weekly basis. The population studied live in Kuala Dura, a riverine village, 5 miles by road from Kuala Brang, 30 miles up-river from the sea and consisting of about 400 inhabitants.

Methods

Approximately 10% of the population was selected, according to convenience and cooperativeness, by the village headman, in such a way that 10% of those aged 12 and over and 10% of those under 12 years were represented. Twice daily visits from Kuala Brang were made by motorcycle to collect the stool specimens which were then processed by methods described in previous reports, with the

addition of plating out on thiosulfate-citrate-bile salts-sucrose medium for *Vibrio parahemolyticus*. The study lasted from 13 March through 6 April, being stopped earlier than planned due to problems of supply and contamination of media. One stool from each participant was taken into polyvinyl alcohol and later screened for helminth ova.

Results

Total population tested	44
Total stools	381
Total for *V. parahemolyticus*	335 (all negative)
Those under 12 years of age (age spread 1-10, mean 4.2)	22
Those 12 and over (age spread 12-63, mean 34.9)	22

None developed significant bacterial diarrhea.

Those with Ascaris ova	8 over 12 (19-48 years)
	6 under 12 (1-5 years)
Those with Trichuris ova	1 over 12
	1 under 12
Those with both	1 over 12
	2 under 12 (6 and 10 years)

Of those selected to participate, 22 fell into 9 family groups of two or more. The Gram stained smears of those were selected for photomicrography and study. The groups are shown thus:

Group	Age	Parasites	No. Stools
1	26	-	7
	3	-	8
	3	-	6
	7	-	2
2	35	Ascaris	8
	2	Trichuris	3
	10	-	3
3	4	-	4
	3	-	2
4	43	-	11
	3	-	7
5	63	-	13
	12	-	5
	3	-	6
6	6	Trichuris and Ascaris	15
	5	Ascaris	11
7	3	Ascaris	3
	1	-	14
8	6	-	6
	1	-	11
9	5	-	15
	1	Ascaris	13

One student was placed in the side-room of the pediatric ward of the University Hospital, Kuala Lumpur, which was concerned with the admission of diarrheal children. Similar techniques were followed, omitting the *V. parahemolyticus* screen. Following plating out and counting of the colonies, the plates were sent daily to USAMRU for definitive identifications to be preformed. The pediatrician in charge cooperated in this part of the study and had enlisted the virology department of the University to study that aspect of diarrhea. Additionally, a blind trial of therapy - antibiotics versus symptomatic care - was incorporated, results of which will not be available for this annual report.

Results

Total cases complete at 30 June	92
Total stools	386
Total cases with pathogen	56
Commonest isolate	*Salmonella spp*, group B
Multiple pathogens in	14
Deaths	4
Age range of patients at first sample	New born to 11 years
Single samples	18

Excluding the single samples, the cases were divided according to the growth patterns of their sequential stools, a random selection made from each group and the Gram smears subjected to photomicrography.

Group	Cases	Stools
No isolates throughout	4	14
No isolates initially, carrier[1] levels later	1	3
No isolates initially, numbers[1] later	1	10
Carriers, remaining so	3	13
Carriers, clearing in hospital	1	10
Carriers, developing numbers	2	14
Numbers isolated throughout	3	15
Numbers, falling to carrier state	2[2]	18
Numbers, falling to no isolates	3	27
Occasional carriers[3]	3	40

Notes: 1. The terms "carrier" and "numbers" refer to the pathogens included in the above cases. These were *Salmonella spp*, groups B C_1 D E_1; *Shigella sonnei* and *E. coli*, serotypes 0119 and 0128. For the present study, no purpose is served in distinguishing between pathogens. The carrier state is defined as stool in which there is no growth in quantitative culture at dilutions of 10^5 or greater, but in which qualitative streaking gives an isolate.

2. One patient died.

3. Those in which one or at most two stools gave an isolate: in two of the cases, different organisms at different times.

The third study group consisted of premature neonates from the Maternity Unit of the General Hospital. Attempts were made to sample each child daily during its stay in hospital, sample collections being made twice or thrice daily. A simpler scheme was followed, omitting desoxycholate citrate and brilliant green agars from the quantitative cultures, where only McConkey agar was used. A total of 106 babies was tested, 689 stools being examined.

Results

The cases were divided into four groups based on the pathogen isolation patterns:

	Cases	Stools	Meconium
No pathogen throughout	30	123	4[3]
Pathogen throughout	23	123[1]	-
Pathogen, becoming negative	16	140	2[2]
Negative, acquiring pathogen	36	299	2[3]
Salmonella typhimurium[4]	1	4	

Thus, 75 of 105 had, at some time, *E. coli* serotype 0119, B14 isolated in significant numbers.

In all instances where a pathogen was isolated, it was *E. coli* serotype 0119 B14, except in one instance of *Salmonella typhimurium*.

Notes: 1. One sample, taken at less than 24 hours post partum, contained the *E. coli* at levels of less than 10^5 per gram stool, with *Serratia* spp as the only other inhabitant, at levels of 10^8 per gram.

2. There was no growth in these meconium stools.

3. Of these 6 meconium stools, 2 showed no growth, 3 had levels of less than 10^6 organisms per gram and one had levels of less than 10^5 per gram. None had pathogen.

4. This child had been abandoned some months before and had been reared by the ward staff. The fact that no other case of *Salmonella typhimurium* infection occurred suggests that the factors affecting spread of *E. coli* were not simply related to poor technique.

Gram stained smears were selected at random from each group thus:

	Cases	Stools
No pathogens throughout	3	20
Pathogens throughout	6	34
Pathogens, becoming negative	3	38
Negative, acquiring pathogens	3	35

In Table 1 is shown the "sequential" nature of the sampling of these cases.

Photomicrography

The gram stained fecal smears selected from each study were photographed by one observer. Photomicrography was performed in a non-selective manner in that the whole smear was rapidly scanned to assess the homogeneity of the preparation and this having been established, a random field was taken for exposure. The only conscious attempt to select was to ensure that those organisms appearing in the field were representative of the distribution seen on scanning. There were occasions on which the Gram smear looked over-decolorised, as judged by morphology and, to ensure uniformity, these slides were fully decolorised and re-stained.

Assessment of Photomicrographs

The transparencies were selected at random, but retaining the sequence for each patient and inserted into slide carriers, the end of one and beginning of the next series being signified by a blank. The first observer defined his own criteria of normal and of infection and, setting his own time for slide inspection, assessed each sequence of stools in turn, noting at the end of each series, the types of change in pathogen load within each series.

The laboratory technician who had been involved in the study over the last 2½ years, the veterinarian who, from many technical briefings to visitors to the unit, had knowledge of the concepts, and three U.S. Army Medical Corps officers stationed temporarily at this research unit, participating in a tropical medicine fellowship, were then invited to assess the slides. All five were given a brief explanation of the diagnostic concepts under evaluation as described in the two previous annual reports. They were then shown six sets of sequential slides containing 7 normal, 3 abnormal, 6 normal, 2 normal, 4 abnormal and 8 normal stool smears. The normals and abnormals were identified to them and this was repeated once while all five examiners mentally established their diagnostic criteria. Slides were then presented in sequences, selected at random by the first observer without reference to whether they were normal or abnormal. There was no communication between the person selecting the slides nor within the panel of observers. Any slide or series could be re-viewed if desired. Observations were noted after each series was shown. In all, 60 human and 3 monkey stool sequential smears were inspected, amounting to 511 transparencies.

Table 1

PATTERN OF SEQUENTIAL STOOL EXAMINATIONS IN ILLUSTRATIVE NEONATE CASES

TYPE	CASE	Pattern (DAYS OF SAMPLING)
NEGATIVE	20	o o o o
	26	[o o] o
	89	o o o o
POSITIVE	24	x x x x
	53	[x x x
	54	[x x x x
	57	x x x x x x x
	59	[x] x x x x x x x x x
	60	[x x]
NEGATIVE TO POSITIVE	37	o o o o δ① x x x x x o
	40	o o o [ ]x x x[x] x x x o
	72	o o o x x x x x
POSITIVE TO NEGATIVE	7	x x x o o o o o x②
	34	x x x x x x x o o o o o o o o o
NEGATIVE	43	o❸ x x x x o

DAYS OF SAMPLING: 1   7   14   21   28   35   42

KEY:
x = E.coli serotype 0119 isolated
o = No pathogen isolated
□ = Antibiotic given
① = No growth in this stool
②❷ = Remained negative in 11 samples over succeeding 28 days
❸ = Meconium

Results

Of the several criteria selected, proportion of Gram negative to Gram positive organisms and the uniformity or non-uniformity of cell shapes were taken as distinguishing features by all observers. Three observers laid emphasis on the scantiness of organisms per slide and the presence or absence of amorphous material. Two observers noted an increase of clumping of organisms in the pathogenic stool smear.

Each of the three monkey stool smears was incorrectly identified as human, despite the flora being generally smaller in size and containing forms not seen in human stool. The scoring of the observers is shown in Figure 2.

The accuracy of evaluations by this group of examiners, presented under the headings of Dura, University Hospital and General Hospital, in a manner capable of analysis, is summarized:

	Agreement with Quantitative Cultures	False Positives	False Negatives	Doubtful	Totals
Dura	85	43	0	4	132
University Hospital	77	38	20	3	138
General Hospital	48	15	25	2	90
Totals	210	96	45	9	360

Discussion

If the X^2 test is used, it may be applied in two ways:

1. If it is accepted that, for the method to be foolproof, only those showing agreement with the quantitative result should be set against those which are false positive plus false negative plus those doubtful, then $X^2_{(2)} = 3.28$.

2. Accepting that a false positive result is allowable, a false negative not allowable and the doubtful results being distributed between agreement and false negative, then, $X^2_{(2)} = 30.89$.

Thus, by applying a rigid yes or no answer, the result is not significant (P less than 0.2) and the method has no value as a screening method. When false positive results are considered acceptable, the method gives a significant correlation with the quantitative stool result (P less than 0.01). This is based on the consistently negative isolations of pathogen from the Dura part of the study. The result of primary plating of stool was vitiated by contamination; the selenite F subcultures, performed at USAMRU, were not. The latter would have demonstrated *Salmonella spp* if present. The same does not hold true for *Shigella spp*. It is recorded that none of the persons under study in Dura reported significant diarrhea during the period.

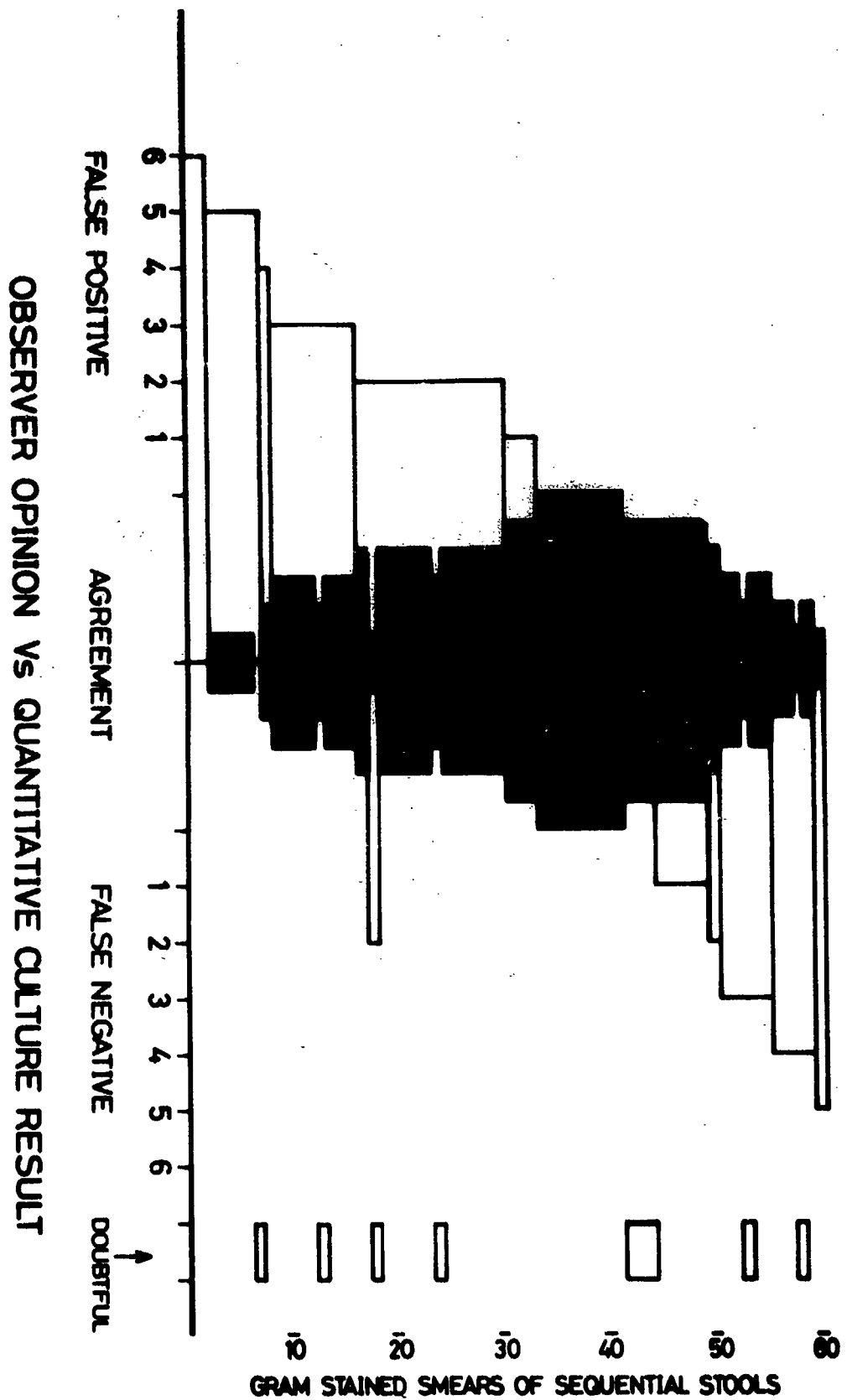OPINION OF 6 OBSERVERS ON 60 SEQUENTIAL STOOL SMEARS COMPARISONS WITH RESULTS OF CULTURE.

Figure 2

It would appear from Figure 2 that, even when criteria are selected individually by the observers, there is some consistency in the patterns of opinion expressed. Cases 1 to 7, 31 to 49 and case 60 show this well and it is interesting to note that there is complete or near complete error in the case of numbers 1 to 7 and number 60. The transparencies in question are to be reviewed by Dr. Kyser without prior knowledge of the quantitative results. Comparison of his with the present results will then be made.

Table 1 illustrates the sampling sequences of the neonate stools selected for photomicrography. Of the neonates sampled and from whom *E. coli* serotype O119 was isolated very few showed signs of disease attributable to this organism. This is at variance with most other experience of enteropathogenic serotypes of *E. coli* in neonate nurseries and raises the question of whether maternal antibody played a significant protective role in these children. It is also worth noting that, despite determined attempts to obtain daily specimens from a population that defecates at least daily, there are large gaps in time between specimens. This was a common experience in both hospitals and illustrates the difficulties in such a study.

Conclusions

Any method used as a screen ideally must contain a minimum possibility of false negative result. This cannot be said for the one under discussion, and, based on the results obtained, there is doubt of its validity.

It must be remembered that the assessments contained here were made on sequences of stools. In the outpatient department the assessment would be on a single specimen, without the benefit of relationship to any preceding or following. This suggests even greater doubt for the validity of results obtained by this method.

Pertussis-like illness

In late Autumn 1971, notice was given of an outbreak of respiratory disease in Kampong Bukit Kemandol, a predominantly aborigine village in Selangor State. It had commenced two weeks previously and, by that time, two children had died. Six of the worst affected were admitted to Gombak Aborigine Hospital, three of whom subsequently died, one in University Hospital, where an autopsy was performed. The symptomatology was of persistent cough with some vomiting; a high fever and a high total white cell count of up to 104,000 per cu mm in one child, and with a differential of 60-80% lymphocytes. Initially the lung fields were clinically clear and those who died did so of a convulsive cyanotic death. The epidemiology of this, confined as it was to younger children mostly in the age range 2-5, together with the findings, suggested pertussis. A determined attempt was made to isolate the organism, using pernasal swabs and Bordet-Gengou agar. Transport of the inoculated medium was possible to the laboratory within an hour. No isolates were obtained from 38 children.

Viral complement fixation tests were performed on paired sera from three of the children. The results show a rising titer to adenovirus. The autopsy indicated a viral interstitial pneumonitis with a secondary bacterial broncho-pneumonia.

Connor, in The New England Journal of Medicine, *283* p. 390, gives evidence for an aetiologic role of adenovirus infection in the pertussis syndrome. It seems valid to question if the epidemic of whooping cough reported by Haug *et al.* in the Annual Report of 1966-67, when only one *Bordetella pertussis* was isolated from 139 specimens, was in fact pertussis and not adenovirus. Further support for a viral aetiology at Kampong Bukit Kemandol is given by the fact that a large number of the children in the affected age group had received pertussis vaccine.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY 30 06 72		REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 30 06 71	4. KIND OF SUMMARY	5. SUMMARY SCTY U	6. WORK SECURITY	7. REGRADING N/A	8A. DISB'N INSTR'N NL	8B. SPECIFIC DATA- CONTRACTOR ACCESS ☐ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		3A062110A831		
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)
Ecological Studies of Mammals and their Involvement in Transmission of Zoonotic Diseases in Equatorial Ecosystems.

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
010100 Microbiology

13. START DATE 10 71	14. ESTIMATED COMPLETION DATE 9 72	15. FUNDING AGENCY	16. PERFORMANCE METHOD

17. CONTRACT/GRANT DADA17-72-G-9350
a. DATES/EFFECTIVE: 10 71   EXPIRATION: 10 72
b. NUMBER:
c. TYPE: Y Grant   d. AMOUNT: 218
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 72	1.0	29.5
	CURRENT 73	1.0	34.5

19. RESPONSIBLE DOD ORGANIZATION

NAME:
US Army Medical Research Unit
Institute for Medical Research
Kuala Lumpur, Malaysia

RESPONSIBLE INDIVIDUAL
NAME: Dr. R. Bhagwan Singh, Acting Director
TELEPHONE: Institute for Medical Research

21. GENERAL USE

20. PERFORMING ORGANIZATION

NAME:
Institute for Medical Research
Kuala Lumpur, Malaysia

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Muul, I., CPT, MSC
TELEPHONE:
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Lim, B.L., M. Sc.
NAME:

22. KEYWORDS (Precede EACH with Security Classification Code)
see continuation sheet

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) Technical Objective: To study factors that determine the degree of activity of zoonoses in various habitats as correlated with distribution, altitudinal affinities, and habitat preferences of host and vector species, and to define potential reservoirs and amplifying hosts among the various mammals on the basis of their population dynamics, seasonality and periodicity of reproduction, litter size and frequency.

To correlate the infestation patterns of ecto- and endo-parasites of mammals, including vectors of zoonotic agents, with ecological factors such as habitats, vertical distribution, periodicity of activity, and diets.

To continue systematic and zoogeographic studies of mammals to determine which are the separate ecologically functional units in the ecosystems studied so that in an epidemiological study only definitive forms among the hundreds of species of mammals need to be of concern.

To continue development of a *species association index* (SAI) for use in epidemiological studies so that investigators may have a probability projection, on the basis of more limited surveys, of faunistic diversity in various "type" habitats in equatorial ecosystems.

To use baseline data from West Malaysia for comparative studies of distribution and activity of zoonoses in nearby areas that comprise "Sundaland". We have received a great deal of encouragement for cooperative work from investigators associated with

DD FORM 1498   PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

DD Form 1498, Research and Technology Work Unit Summary,
Item 23 Continued:

the SEAMEO BIOTROP program in Indonesia, from the Director and staff
of the Applied Scientific Research Corporation of Thailand, and
from appropriate researchers in Sabah.

24.(U) Approach: The methods used for collecting ecological and
epidemiological data on mammals and their involvement in transmission
of zoonotic diseases have been discussed in the last several annual
reports (USAMRU Annual Research Progress Reports 1968-1971). During
the year covered by this report several new lines of investigation
were begun. The main one of these was the establishment of a new
study area in the Bukit Lanjan area. The previously established
vertical stratification study areas with canopy transect walkways
are located at the headwaters of S. tambul, near Bukit Resam. A
more detailed map of the *Orang Asli* (aborigine) village and the
specific study sites is shown.

25.(U) Progress: Data from field studies are beginning to support
the conceptual role of medical ecology (Muul, 1970, Science, 170:
1275-1279; Abu Bakar bin Ibrahim, Muul, and Lim, 1971. Mimeographed.
Institute for Medical Research). Understanding the enzootic transmission
cycles of zoonotic pathogens in natural hosts lends predictive value
to anticipating the epidemics in unnatural hosts, such as man, under
specific circumstances. For example, the habitat of greatest enzootic
activity of scrub typhus *(Rickettsia tsutsugamushi)* in Malaysia is the
forest rather than the classical "scrub" habitat previously described.
This was found to be the case for areas tested both in West and East
Malaysia (Sabah). In both areas arboreal rodents were not involved, not
even as the postulated nidus or primordial source for infection (Audy,
1961, in May, J.M., ed. Studies in Disease Ecology, Hafner, N.Y.).
Seasonal effects appear to be operant, which may obscure the results of
short-term surveys. Species respond differentially to the infection as
predisposed by their ecological niches. In survey trapping, many
species of arboreal mammals are missed. These are actually shown to
be abundant when other collecting methods (such as capture by hand from
arboreal tree cavities) are employed. Studies with the aid of the
canopy transect walkway system (Muul & Lim, 1970, Science, 169: 788-
789) have shown that there is little overlap in specie' diversity in
the forest canopy and that on the ground. Rates of parasitization,
such as with *Plasmodium* differ also according to vertical zonations.
Forests that appear similar differ markedly in their species diversity
depending on their age and history. Prevalences of various zoonotic
agents also differ in various habitats (e.g. Muul, Lim, and Yap, 1970.
S.E. Asian J. Trop. Med. & Publ. Hlth., 1(3): 418-419). Additional
data for seasonality studies of ecological phenomena and temporal
distribution of zoonotic pathogens are being accumulated and analyzed.

Item 22

Medical Ecology, ecological niches, vertical zonation, species
diversity, zoonotic pathogens, *Rickettsia tsutsugamushi*, habitats,
tropical rainforest, seasonal phenomena, serological results, canopy
transect walkways, blood parasites, trapping, West and East Malaysia,
enzootic transmission, forest canopy, arboreal mammals.

ECOLOGICAL STUDIES OF MAMMALS AND THEIR INVOLVEMENT IN TRANSMISSION OF
ZOONOTIC DISEASES IN EQUATORIAL ECOSYSTEMS

Background

The modern concept of epidemiology has been broadened by the
emerging disciplines of medical ecology and zoonoses. Traditionally,
epidemiological studies have been primarily anthropocentric. The
focus has been largely on disease epidemics as they occur in human
populations. Because of the increasing awareness that a number of
human diseases involve animals as reservoirs of infection, the broadened
concept of epidemiology must necessarily include the study of endemic
cycles of diseases with regard to the mainstream of vector and host
biology.

Epidemiology and medical ecology had been considered previously
as essentially synonymous (Audy, J.R., 1965). However, medical
ecology has been defined recently as the study of those ecological
relationships between organisms and their environment that are of
medical significance (Abu Bakar *et al.*, 1971). The environment includes
physiochemical and biotic factors. The latter include all other
organisms in the environment, including zoonotic pathogens and factors
that predispose natural and unnatural hosts to infections with these
pathogens.

Morbidity and mortality statistics for zoonoses are not always
impressive and usually do not run to large proportions, since many of
the pathogens which are potential zoonoses involve man only occasionally
or accidentally. Thus, the zoonoses often present a problem of
apparently small magnitude. Furthermore, most studies of zoonoses are
confined to those involving domestic animals hosts leaving unrecognized
the potential hazards presented by a large group of others which are
known to involve sylvatic hosts, particularly rodents. The potentials,
therefore, for an epidemic involving a given zoonoses are apparently
unrecognized and their assumption of epidemic proportions is
unpredictable. This is especially true of a number of fevers reported
as of unknown origin.

The most frequently encountered zoonotic diseases appear to be
caused by common endemic pathogens of man's closest relatives, the
mammals (Abu Bakar *et al*, 1971; Famatiga, E., 1971). The involvement
of man in a zoonotic disease cycle may be accidental or incidental in
regard to the normal endemic transmission cycle of a pathogen.
However, the involvement of mammalian hosts should be predictable.
Since epidemiology is the study of epidemic disease, epidemiological
studies are usually not begun until an epidemic occurs. By the time an
epidemic erupts in the human population, the enzootic factors that were
important in leading up to the epidemic may no longer exist.

The recognition of the cryptic nature of the zoonoses has brought to light the importance of determining the characteristics of the endemic transmission cycles of zoonotic pathogens. Many of the various aspects of the host biology, such as distribution, feeding habits, periodicity of reproduction, population dynamics and behavioral factors, may have important bearing on disease transmission (Muul, I., 1970). These various aspects of host biology are within the purview of medical ecology.

A comprehensive approach to human disease prevention and control must comprise more than only the study of epidemics in man, which has been the case in many epidemiologic studies. Emphasis should be placed on ecological studies of the enzootic disease cycles in natural sylvatic hosts in order to provide a deeper insight into the natural transmission cycles and to derive a predictive capability. Studies in medical ecology are complementary to and necessary for epidemiological investigations, particularly in providing pre-epidemic clues (Abu Bakar *et al*, 1971).

The public health impact and the socio-economic consequences of the zoonoses have been recognized (World Health Organization, 1969). The magnitude of losses resulting from zoonoses should be measured not only in terms of loss in manpower resulting from disabilities and diseases such as among military personnel, but also in terms of hunger and malnutrition resulting from losses in animal protein from the diet of people who form the political base that is important in supporting the military operations.

Objectives

To study factors that determine the degree of activity of zoonoses in various habitats as correlated with distribution, altitudinal affinities, and habitat preferences of host and vector species; and to define potential reservoirs and amplifying hosts among the various mammals, on the basis of their population dynamics, seasonality and periodicity of reproduction, litter size and frequency.

To correlate the infestation patterns of ecto- and endo-parasites of mammals, including vectors of zoonotic agents, with ecological factors such as habitats, vertical distribution, periodicity of activity, and diets.

To continue systematic and zoogeographic studies of mammals to determine which groups form the separate ecologically functional units in the ecosystems studied, in order that only certain key forms among the hundreds of species of mammals need to be of concern in any restricted epidemiologic study.

To continue development of a *species association index* (SAI) for use in epidemiological studies so that investigators may predict or project faunistic diversity in various "type" habitats in equatorial ecosystems.

To use data from West Malaysia for comparative studies of
distribution and activity of zoonoses in nearby areas that compose
"Sundaland". We have received a great deal of encouragement for
cooperative work from investigators associated with the SEAMEO
BIOTROP program in Indonesia, from the Director and staff of the
Applied Scientific Research Corporation of Thailand, and from
appropriate researchers in Sabah.

Approach

The methods used for collecting ecological and epidemiological
data on mammals and their involvement in transmission of zoonotic
diseases have been discussed in the last several annual reports
(USAMRU Annual Research Progress Reports 1968-1971). During the
year covered by this report several new lines of investigation were
begun. The main one of these was the establishment of a new study
area in the Bukit Lanjan area (Fig. 1). In Figure 1 the main
roads leading to Kuala Lumpur and Damansara (toward south) are
shown. The previously established vertical stratification study areas
with canopy transect walkways are located at the headwaters of S.
Tambul, near Bukit Resam. A more detailed map of the *Orang Asli*
(aborigine) village and the specific study sites is shown in Figure 2.

Scrub Typhus: A study of seasonal prevalence and transmission
of scrub typhus rickettsiae *(Rickettsia tsutsugamushi)* in various
habitats was begun in August 1971. This project developed as a
result of earlier survey work reported on in the 1971 Annual Report.
The previous data seemed to show that the activity of the transmission
cycle varied according to habitats. During this initial survey
collecting was done in various areas over a period of two years
without equal emphasis on each season. Consequently, the role of
seasonality was not adequately demonstrated. Accordingly, four
habitats were selected in which grids were established for
continuous trapping for linear studies (as shown in Figure 2). One
group of traps was located in the *Orang Asli* village, with traps
placed in or in the immediate vicinity of houses. Another group of
traps was set along the border of the forest and the clearing under
the high voltage lines. This was considered as edge habitat or
"scrub", a transition type habitat. The clearing under the
electrical power lines has been maintained by Government workers by
burning, resulting in an overgrowth of *lalang* grass, *Imperata
cylindrica.* The latter composed the third habitat as shown in
Figure 2. The fourth trapping grid was established in the forest.

Because it was established during the initial survey work that
species were variously predisposed to showing active rickettsemia,
as demonstrated through isolation attempts, only a few indicator
species were chosen for the linear studies. In the village
habitat, the house rats, *Rattus rattus diardii* and *Rattus exulans*,
and the scrub and field rats, *Rattus tiomanicus* and *R. argentiventer*
were followed. These species were also tested as they occurred in
the other habitats. In the forest, and sometimes in the edge

BUKIT LANJAN STUDY AREA

1:67,000
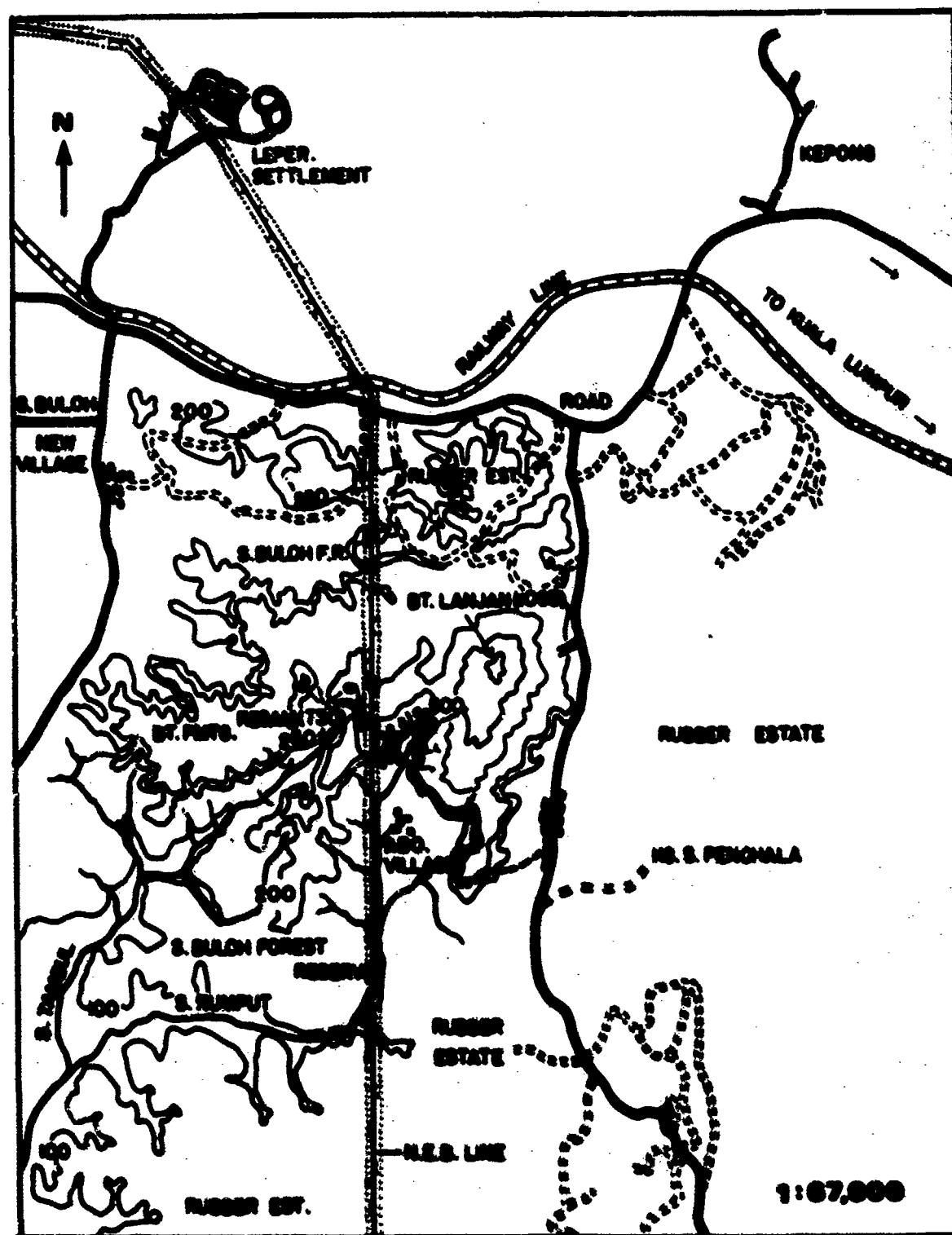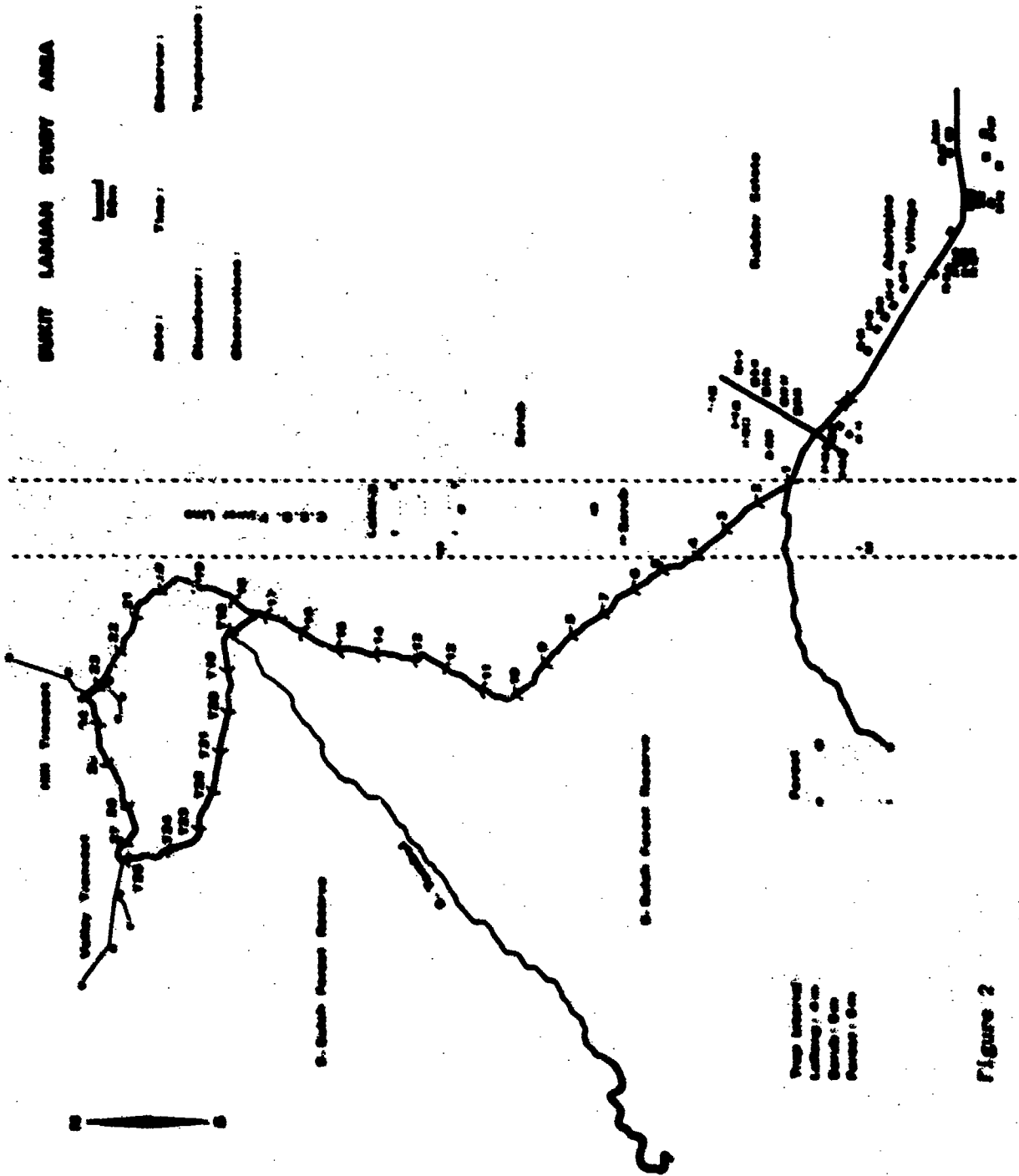
Figure 2

habitats, the indicator species used was *Rattus sabanus*. The forest grid yielded the fewest rats, and the numbers of *R. sabanus* were supplemented by those captured in conjunction with the vertical stratification studies farther in the forest (i.e. Hill and Valley transects).

In January an area of forest which included the forest trapping grid was cut during timbering operations. The forest grid was re-established deeper in the forest near the Valley Transect. After the timber cutting was halted a supplementary grid was re-established in the cut-over area to measure the effects of the cutting on the rodent population and their involvement in the rickettsial transmission cycle.

In conjunction with the small mammal studies, the Bukit Lanjan study area was used by the Department of Acarology for ecological field studies of the vector mites (see section on Acarology). In addition the Department of Acarology undertook studies of vector mites collected in the laboratory from the mammals brought in from the field by the Department of Ecology.

The mammals were brought into the laboratory five days a week. They were marked by toe-clipping, examined for ectoparasites, weighed and measured for age classification, and bled for isolation attempts and serological tests. Blood (whole) was injected immediately into white mice by personnel of the Department of Rickettsiology. Vector mites (chiggers) were collected by personnel of the Department of Acarology. A data card was maintained for each animal. The animals were released the following day at the point of capture after they had been under observation over night to determine the immediate effects of the anasthesia and bleeding procedure.

Seasonality Studies: Since our previous data had indicated that cyclic events in animal populations do not necessarily follow the calender year, as is most often the case in temperate climates, several lines of investigation were begun or amplified to better understand these phenomena. Many of the mammalian species depend on fruit, nuts, or other plant products for food. Thus, phenological studies had been conducted and were continuing to attempt to establish bases for correlations with events in animal populations that could account for changes in size and age structure of populations. These studies were expanded in 1971-72 to include trees along the trail to the vertical zonation study area where the canopy transects are located. The trail leading up to the transects was marked at 50 meter intervals (Fig. 2) for easy determination of the source of fallen fruit which were collected daily, five days a week. This work is now being done in conjunction with personnel of the Forest Research Institute (FRI), under the supervision of a botanist, Dr. Francis Ng, who is also interested in additional, more detailed phenological studies. Thus, unusual research opportunities are afforded also to the FRI to conduct studies which were not possible before, having to do not only with phenology, but with more general questions concerning forest ecology and silviculture, as well.

Small surveys of mammals and scrub typhus vectors were conducted
by the Departments of Ecology and Acarology in Indonesia (Java) in
September, 1971. These results are summarized in the Acarology Section.
Also, the results of the serological survey of mammals in various
habitats in East Malaysia (Sabah) have been completed and summarized
in this report. This field study was performed in May 1971 and was
partially summarized regarding the mammalian data in the 1971 Report.

Review of Research - General (1968-1972)

In equatorial ecosystems with a great amount of rainfall, primary
forests grow to great heights. Emergent trees may be 60 meters tall
or more. Beneath the emergents the canopy is nearly complete, in
which small mammals are uniquely adapted for arboreal life. In West
Malaysia many of the 200 species of mammals may be found only in such
habitats and have not been collected in scrub forests or, in some
cases, rarely even in fairly mature secondary forests. In the primary
rainforests mammals can be grouped into categories of arboreal,
semiarboreal, and ground species. Trapping and observation of
arboreal or canopy species has been very difficult. A canopy transect
system constructed of suspended walkways has aided in capture and
observation of canopy species. Special capture methods, such as
collection from arboreal nest cavities, have yielded information about
the non-trappable portion of the mammalian canopy populations.

Although trapping procedures sample only a portion of the small
mammal populations, the numbers of species captured on the ground and
in the canopy are approximately equal. There is little overlap in the
species captured at the two levels. The weight distributions of the
canopy and ground populations differ. At both levels a few species
predominate the collections, while the remainder are much scarcer.
The portion of the canopy mammals which seldom or never entered the
traps, was larger than the portion of ground species which seldom or
never entered the traps. The group of ground species was dominated
by murids, but included some sciurids. The canopy species were mostly
sciurids, but included some murids. Diurnal canopy sciurids and some
of the canopy murids constructed "outside", or dray-type nests.
Nocturnal sciurids and the remainder of the arboreal murids construct
nests in hollows of trees. In these there seemed to be interspecific
competition with others of similar size and in some cases with birds,
scorpions, ants, bees, or spiders. Ground sciurids and murids live
mostly in burrows, but *Rhinosciurus* and *Tupaia* construct nests of
twigs, leaves, and stripped bark at bases of trees or in shrubs.
Niches seem to be partially exclusive on the basis of use of dead or
live trees for nests, height of nests, size of opening to nest
cavity, and species of trees utilized. Diets were observed to vary in
species of similar size; also the periodic use of the environment
varied. Modes of locomotion, leading to differential ability to
utilize certain portions of the habitat and various types of habitat,
seemed also to distinguish niches.

The predisposition of various species to acquiring infections seems to depend to a large extent on their ecological niches. The ecological niche of a species is its role in the total ecosystem. Thus, on the basis of knowledge of the niches of various species an epidemiologist should be able to assess the potential of the species for involvement in a given zoonosis, singling out certain ones so that not all of the hundreds of species of mammals in a given region need to be of concern.

Review of Research - Specific (1972)

During seven months of the period covered by this report, the principal investigator was transferred to Washington, D.C. In his absence the associate investigator Mr. Lim Boo Liat directed and supervised the ongoing linear studies. This was a critical period in some ways as the forest at Bukit Lanjan where several studies are in progress had been contracted for timbering. Fortunately, an agreement was reached to spare some of the forest, including the areas where the vertical stratification studies involving the canopy transect walkways are in progress.

Vertical Distribution of Mammals and Scrub Typhus: Figures 3 and 4 are a preliminary summarization of the mammalian data collected in conjunction with the canopy transect walkways system. The data are pooled from both the Hill and Valley transects. The differences in the involvement of mammals, classified as canopy or ground dwellers, in transmission cycles of scrub typhus rickettsiae were demonstrated in the 1971 Report. In summary, the canopy animals were not found to be involved in the scrub typhus rickettsiae transmission cycle. The present data give a quantitative classification of the various small mammals captured by traps at the two levels within the forest. Of major ecological note pertaining to epidemiological studies is that this collecting procedure, which is often the only one used, indicates that although the numbers of species at each level is large, relatively few species dominate the samples numerically. With a single collecting method it is not possible to establish whether these samples actually represent the true relative abundance of the species. Epidemiological surveys based on trapping results alone may be biased and important species may be missed or their abundance underestimated. Several species were caught in fair numbers both in the canopy and on the ground and appear to range between the two levels, e.g. *Sundasciurus tenuis, Tupaia glis,* and *Lenothrix canus* (formerly *Rattus canus*).

Habitat Distribution of Mammals, Scrub Typhus, and other Pathogens: The last several Annual Reports have summarized general differences in species compositions of mammals in various habitats. Specific cases of differences in infection rates with parasites, such as blood protozoa and scrub typhus rickettsiae, have also been demonstrated. In this report data are summarized in detail to show habitat differences in mammalian species diversity.
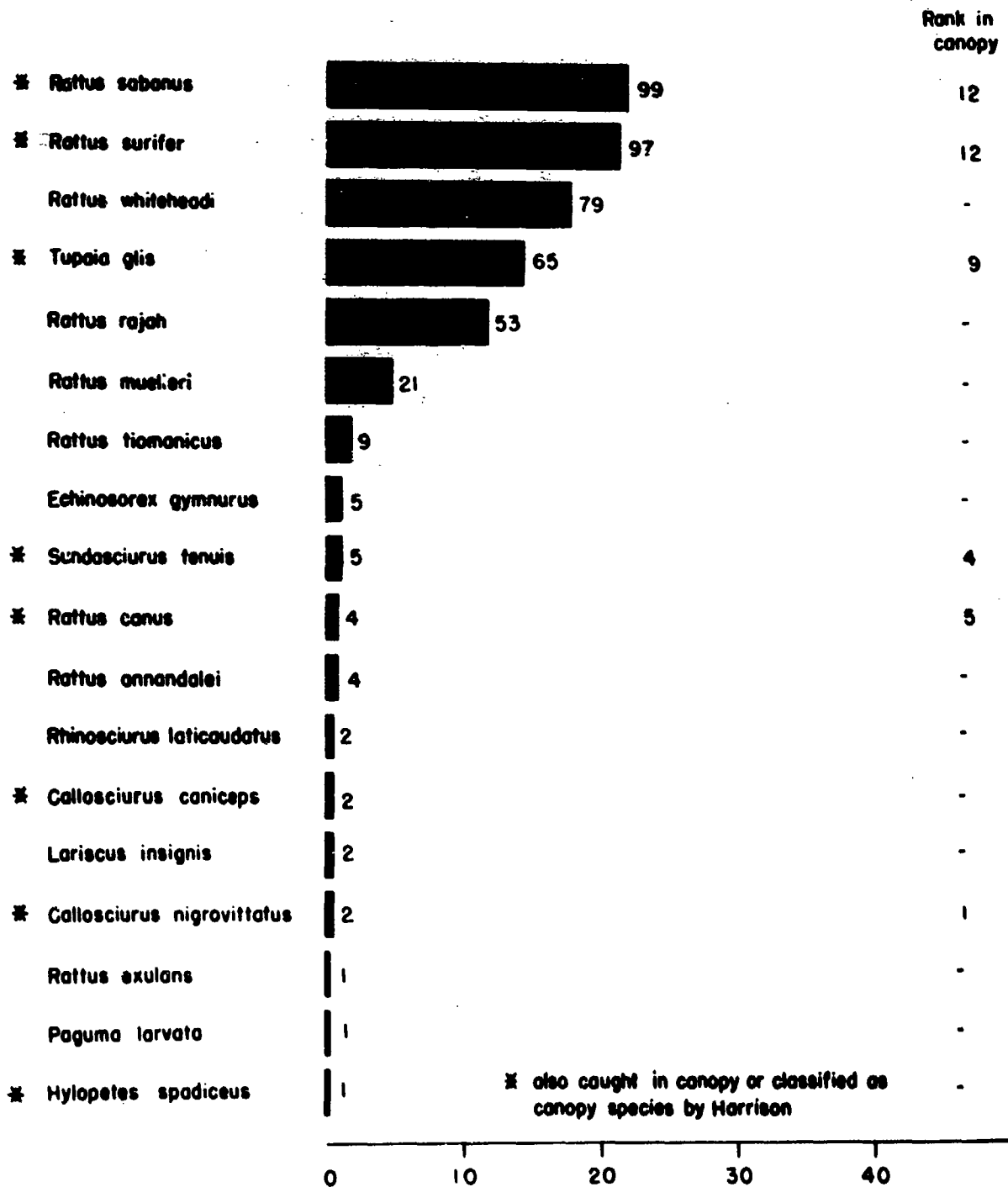
GROUND

Figure 3   PERCENT OF TOTAL CATCH
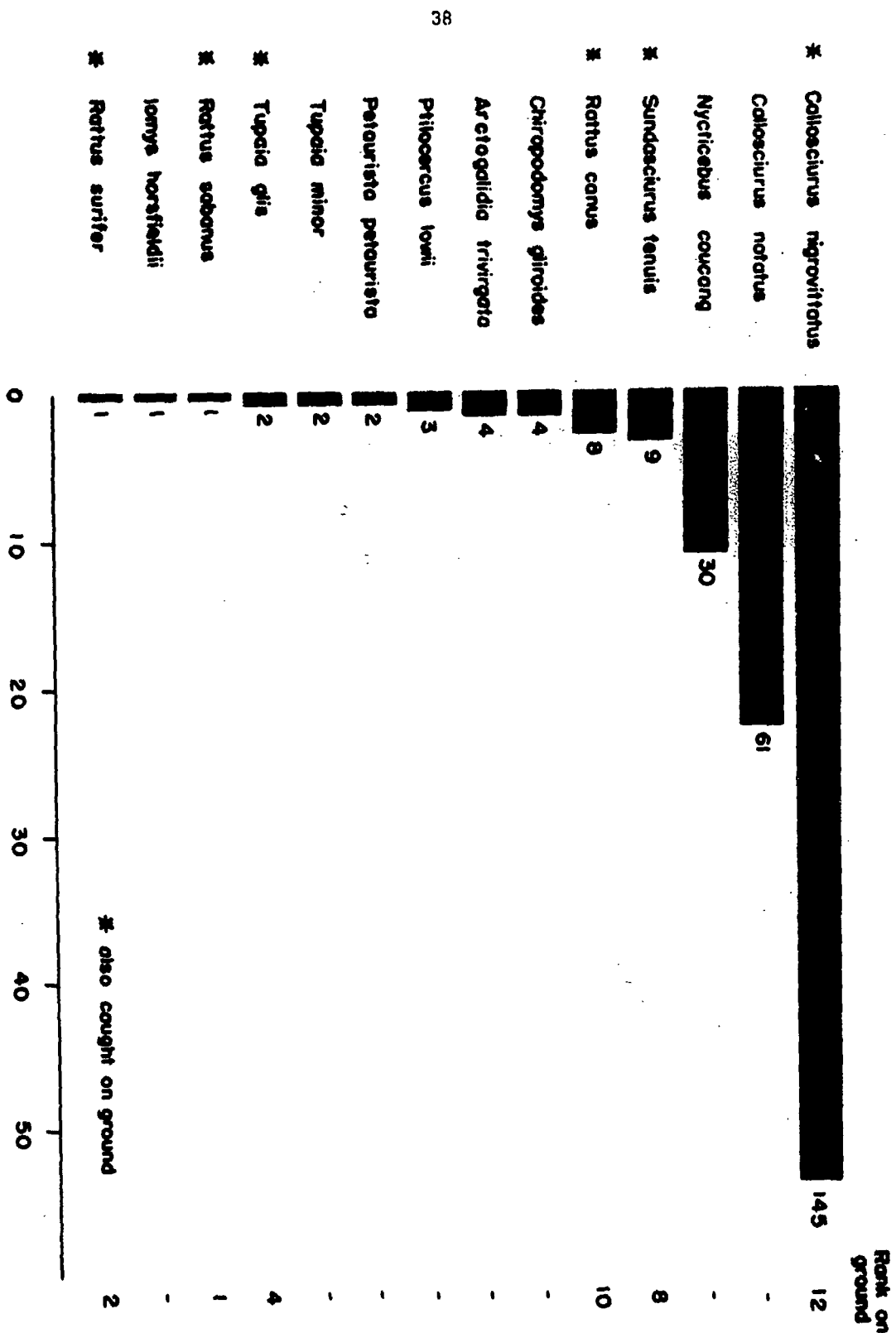
Figure 4   PERCENT OF TOTAL CATCH

* also caught on ground

(a) *Mostly Primary Forest vs. Mostly Secondary Forest:* Two forest areas were compared which differ only slightly in terms of their general appearance. Both areas have been partially timbered in the past. The data for Bukit Lanjan forest appear in Figures 3 and 4. This area is mostly primary forest which was selectively logged about 30 or 40 years ago. The second area is located near the Subang Airport and has been logged much more recently and to a much greater extent, although many relict trees of the former primary forest still stand among the regenerated growth. The samples of mammals collected in 1970 and 1971 in the Subang area from these recently logged forests appear in Figures 5 and 6. The trapping on the ground level was done in exactly the same way, with traps placed in pairs: one in a tree and one on the ground below, and with the same types of traps as were used in Bukit Lanjan. Unlike in Bukit Lanjan, where the numerically dominant ground dwelling rat was *Rattus sabanus* (Fig. 4), the numerically dominant rat was *Rattus annandalei*, which ranked eleventh on the list of species from Bukit Lanjan. Other marked differences were demonstrated in the numerical ranks of *Rattus surifer*, which was second only to *R. sabanus* in Bukit Lanjan, but scarce in the Subang area, whereas *Rattus rajah*, a close relative, ranked nearly equally in the two areas. *Rattus muelleri* was relatively more abundant in the Subang area than in the Bukit Lanjan forest. Two species of squirrels, *Callosciurus prevostii* (Fig. 6) and *Sundasciurus hippurus* (Fig. 5), appear on the Subang list, but were never caught nor observed in the Bukit Lanjan forest. The canopy species are not exactly comparable in the two areas, in that although traps were placed in pairs, one in a tree and one on the ground, the great heights were not often attained in the Subang forest as were possible to reach with the canopy transect walkways in the Bukit Lanjan forest. Nevertheless, among those canopy species captured (Figures 3 and 6) marked differences can be seen. The numerically dominant squirrel in the Subang area was *Callosciurus notatus*, which was caught at a ratio of only about 1:2.5 compared with *Callosciurus nigrovittatus* at the Bukit Lanjan area. Because most of the traps were not placed at any great height, a number of semiarboreal rats were caught in the trees (mainly *R. sabanus, R. annandalei, R. muelleri, R. rajah*). Their relative degree of arboreality can be evaluated by comparing the mean heights (in feet), the ranges of heights at which they were captured, and the per cent of the catch of the species that was obtained in the trees (given in the right hand column of Fig. 6). *R. sabanus* appears to be the most arboreal among these (see also Lim, 1970). In addition to the few squirrels (including *Sundasciurus lowii*) that were caught in Subang and not in Bukit Lanjan, an arboreal rat, *Pithechir parvus* appeared only in the Subang samples. On the other hand, *Nycticebus coucang*, a primate, was scarce in the Subang samples, but abundant in Bukit Lanjan. In summary, these data show that even slight differences in the habitat appear to correlate with significant differences in the fauna (a forest is not a forest, is not a forest).

(b) *Forest vs. Edge Habitats ("Scrub"):* In last year's report data were presented to demonstrate differences in habitats in regard to prevalence of scrub typhus rickettsiae *(Rickettsia tsutsugamushi)*.

GROUND (N=38I)

Rattus annandalei — 90
Rattus sabanus — 88
Rattus muelleri — 59
Rattus rajah — 41
Rattus whiteheadi — 27
Rhinosciurus laticaudatus — 20
Tupaia glis — 18
Lenotherix canus — 10
Rattus tiomanicus — 9
Echinosorex gymnurus — 6
Callosciurus notatus — 5
Sundasciurus tenuis — 3
Rattus surifer — 2
Sundasciurus hippurus — I
Tupaia minor — I
Sundasciurus lowii — I

Figure 5   Percent of Total Catch   Percent of Special Catch

10 20 10 20 30 40 50 60 70 80 90 100

TREES (N=383)

Figure 6

Relatively undisturbed forest areas appeared to have higher iso`.tion
rates of rickettsia from small mammals than did the more disturbed
areas, such as edge habitats (commonly called "scrub") (See both
Ecology and Rickettsiology sections in the 1971 annual report).
Further analyses of these data have shown that there are other
influences as well, such as, seasonality and the species
compositions of the samples. There was no marked fluctuation in the
monthly isolation rate during the early phase of the collections in
the forest habitats, i.e. November 1969 through April 1970 (Fig. 7).
Early during this period the isolation rate (blood) from edge habitats
and regenerating vegetation ("scrub") was lower than that in forest
habitats. By June 1970 the isolation rate in the "scrub" habitats
increased and approximated that in forest habitats. The attempted
isolations in the following period, after October 1970, were done
through testing kidney samples. From October through November the
rate of isolations was higher than that obtained previously (Fig. 8),
but the rate dropped again after December, this time in both forest
and edge ("scrub") habitats. The numbers on top of the figure
represent sample sizes (those in parentheses in Figure 8 represent
blood isolation attempts while most of the samples used during that
period were from kidneys). In summary, there seem to be fluctuations
in isolation rates during different times of the year, but in subsequent
years the high "season" did not occur at the same time of year.
Several years of additional data would be needed to establish the
actual temporal pattern. The isolation rates for individual species
are given in the Rickettsiology section in this report and also in the
1971 Report.

The serological results indicate that in the period of November
1969 through about June 1971 the rickettsial parasites were
generally more active in the forest habitats (Fig. 9). However,
after July 1970 this trend changed toward less disparity between the
habitats (Fig. 10). Nevertheless, the peaks in isolation rates came
at times of relatively low serological rates.

During the period of October 1969 through September 1970 the
overall rickettsial isolation rate (blood) from the forest habitats
was 23% (N=259) while that in the edge habitats ("scrub") was about
10% (N=336). From October 1970 through August 1971, the isolation
rate (kidney) from animals caught in the forests was 21% (N=276) and
the rate in those from edge habitats was 13% (N=587). During the same
period October 1969 through September 1970 the serologically positive
animals composed 80% of those tested (N=206), while positives among
those from edge habitats composed 42% of those tested (N=298). The
following year (October 1970 through July 1971) the rate for
serologically positive reactors was 64% (N=543) in the forest samples
and also 64% (N=783) in the samples from edge habitats. These results
also seem to indicate that if there is a seasonal pattern, it is
probably not the same in different habitats. Whatever ecological
factors are influencing the transmission (or synchronous recrudescence
of infections) seem to be different in these habitats. Year to year
differences also are apparent.

Forest Scrub

6 5 51 30 6 29 6 138 27 22 47 22 37 28 17 7 85 23 14 20

O N D J F M A M J J A S
1969 1970

% Rickettsial isolations from Blood
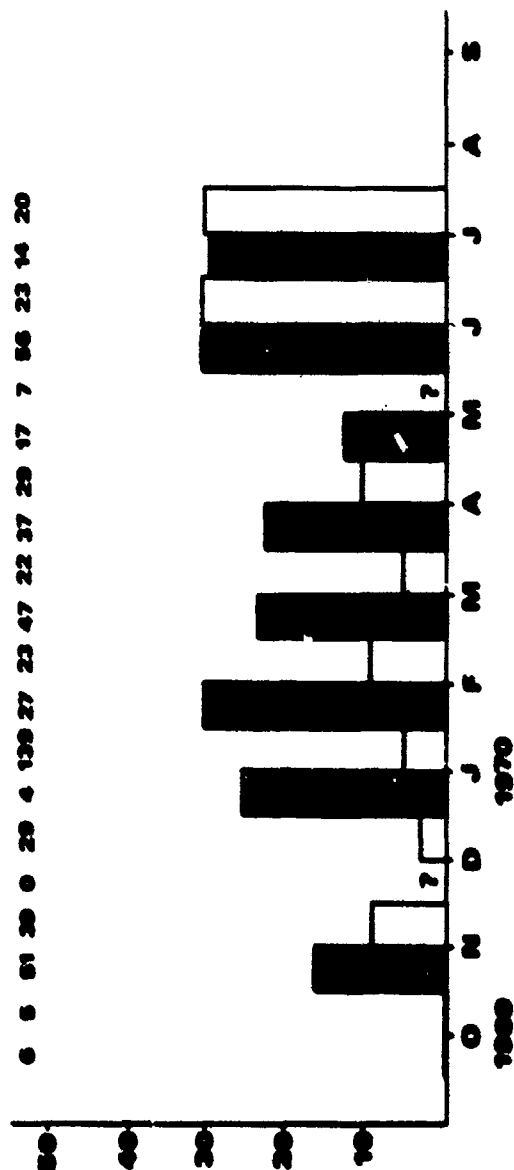
Figure 7

Note: Hatched bars indicate small samples; question marks indicate no samples or small samples with no positives.

38 63 65 84 67 10 22 39 29 46 4 288 29 67 128 65 138 2 83 0 11

O N D J F M A M J J A S
1970 1971

% Rickettsial isolations from Kidneys
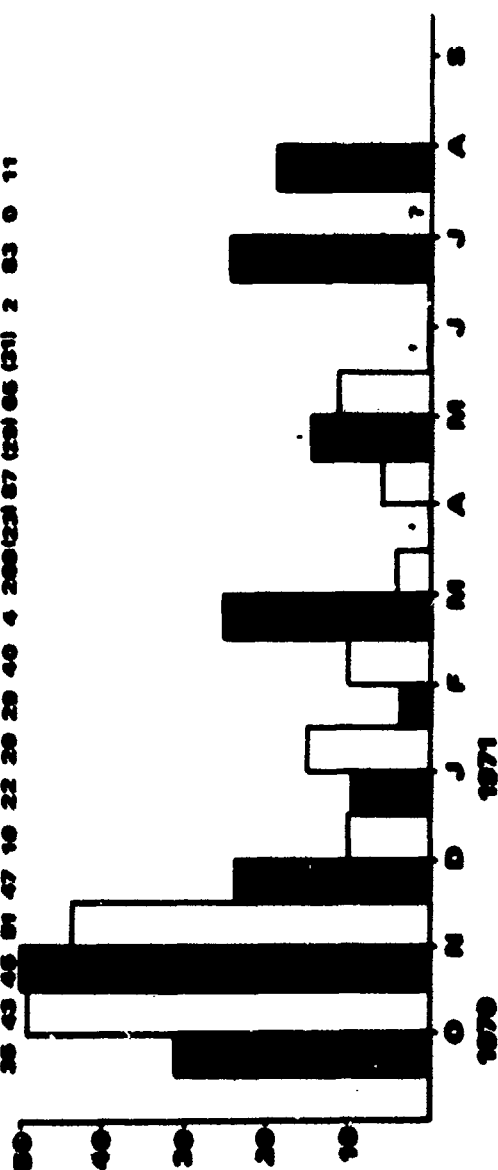
Figure 8

% Positive Serologically

Figure 10
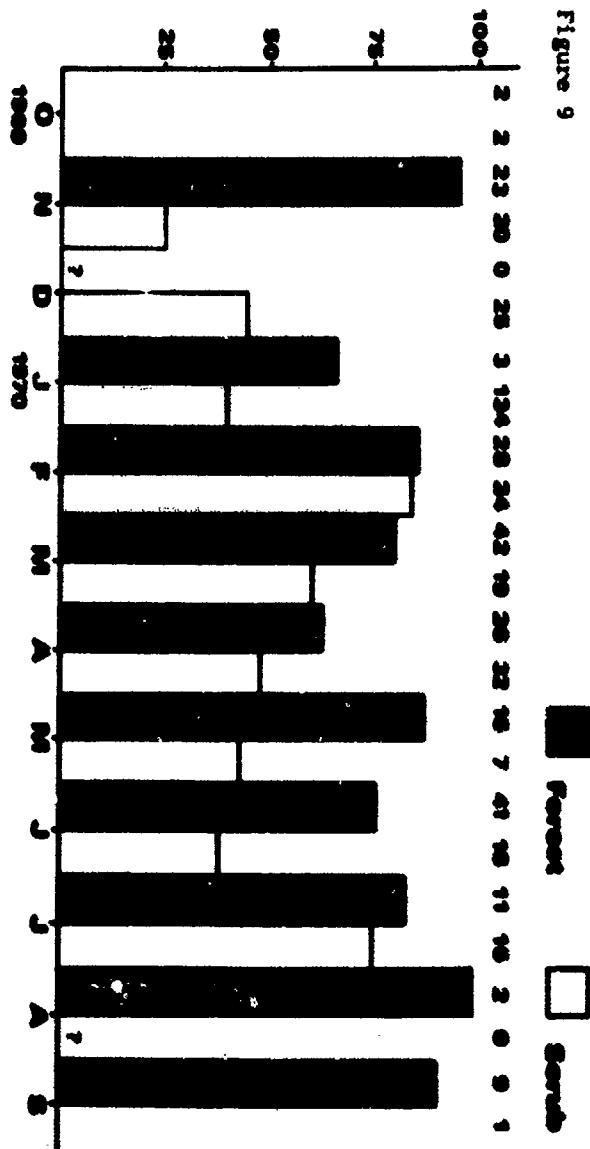
Figure 9
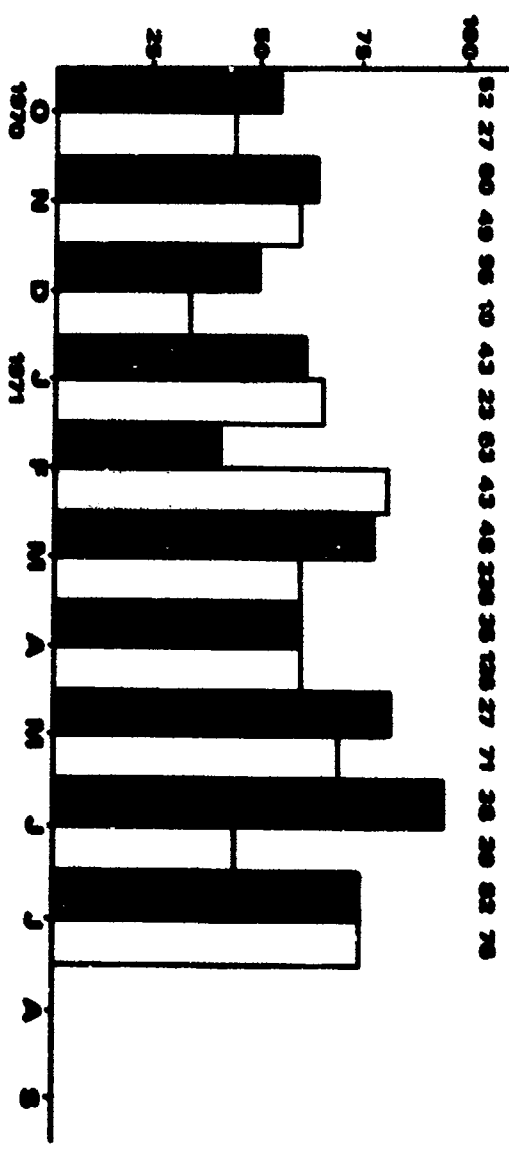
The months with the highest isolation rates in edge habitats included in their respective samples a high proportion of typical forest species, such as *Rattus sabanus*, thus introducing still another variable into the system, i.e. the species composition of the sample. *R. sabanus* among the various species tested usually yielded a high proportion of positives in isolation attempts, as well as positive serological reactors. For this reason prospective studies now underway include only selected indicator species for each habitat.

Seasonal Studies of Habitats at Bukit Lanjan: The data from the prospective linear studies involving four habitat types at Bukit Lanjan are still in a preliminary stage. Rickettsial isolation data are available only through early 1972. Through that time period 656 blood samples for isolations and 771 for serology from four indicator species from the various habitats have been analyzed (Table 1). The selected species were not always confined to a single habitat. Also their species specific response to infections with *R. tsutsugamushi* are not yet known. Therefore comparisons cannot be made without some possible bias until responses to infection in these species can be quantified in the laboratory. Nevertheless some of the species were captured in more than one habitat and thus allow some comparisons of habitats within species. For example, *R. argentiventer* was caught in significant numbers in both the village and in lalang. In the village this rat was in the minority which suggests that it uses this type of habitat relatively infrequently. The village has some fruit trees, coconut trees, brush, herbaceous vegetation and some grass. Some of the ground is bare, packed clay, especially around houses. This type of habitat would be called mostly "scrub" if the houses were absent. The rickettsial isolation rate in *R. argentiventer* in the village was lower than the rate in lalang. In *R. tiomanicus* the rickettsial isolation rates in these two habitats were nearly equal, however, the sero-positives in the village were lower than in the lalang or in the "scrub".

It appears from these data that transmission of rickettsiae to rats is occurring at a high rate in the lalang. Perhaps the close proximity of the lalang to the "scrub" habitat and the apparently free movement of the rats between these habitats (the typically "scrub" rat, *R. tiomanicus* was actually more frequently caught in the lalang than in the "scrub"), the results may be somewhat obscured in regard to habitat differences in scrub typhus transmission to rats. Since very few "scrub" or lalang rats were caught in the forest, yet a high yield of rickettsial isolations was obtained in the forest, the results support the original thesis that the enzootic transmission of rickettsiae to rats is very active in the forest. It is clear that the transmission is active also in the lalang and less so in the village. But, the results for the "scrub" habitat, although not an unusually high isolation rate was obtained, tend to be obscured by the close proximity of this study plot to the lalang and the apparently free movement of rats between this plot and the lalang. Therefore, we are planning to modify the techniques somewhat to attempt to reduce some of these variables beginning in October 1972.

Table 1

Scrub typhus (*Rickettsia tsutsugamushi*) isolations and serological data in four selected species of rats in four types of habitats in West Malaysia (August 1971 - February 1972)
(N) = Number of samples tested; I = Percent of rickettsial isolations; S = Percent of serological pos.

Habitats	Species								Total	
	R. exulans		*R. argentiventer*		*R. tiomanicus*		*R. sabanus*			
	I(N)	S(N)	I(N)	S(N)	I(N)	S(N)	I(N)	S(N)	I(N)	S(N)
Village	14(83)	17(106)	24(33)	37(38)	24(140)	26(151)	-	-	21(256)	24(295)
Lalang	31(13)	14(14)	46(56)	60(73)	27(158)	45(175)	-	-	32(227)	47(262)
Scrub	0(3)	0(3)	50(12)	19(16)	26(47)	45(49)	60(5)	53(34)	31(67)	42(102)
Forest							31(106)	38(112)	31(106)	38(112)
Total	16(99)	16(123)	40(101)	47(127)	26(345)	38(375)	32(111)	42(146)	27(656)	37(771)

Definite differences in the mammalian species compositions were also observed (Fig. 11). *Rattus surifer* seemed to be a significantly abundant species in all habitats, but other forest species were scarcer in edge habitats and lalang than in the forest. This species could have been a good candidate for following all of the habitats with a single species were it not for the fact that it seldom is infested with chiggers. The composition of the catch was also observed to change through the trapping period. Partial logging was begun in the forest in January 1972. Concurrent with this, but not necessarily caused by this, was a relative increase in the proportion of certain species caught, notably *Rattus annandalei* in the forest and forest edge and *R. tiomanicus* in the edge habitat and in lalang (Fig. 11). Such shifts in the apparent species compositions may also be associated with, or even influence, the prevalence of pathogens.

It is notable that even though some species ranged through several habitats their rickettsial isolation rates were about the same whever they occurred (with the exception of *R. argentiventer* in the village):

HABITAT

Species	Edge	Lalang	Village
R. tiomanicus jalorensis	26% (47)	27% (158)	24% (140)
R. argentiventer	50% (12)	46% (56)	24% (33)

Other Parasite Patterns: Further data have substantiated the earlier observations of habitat differences in rates of infection with blood parasites (Annual Research Progress Report, 1970).

Trap Response and Population Estimates of Hosts: When one compares the weight distributions of animals caught on the ground and in the canopy in association with the canopy transect system at Bukit Lanjan differences are immediately apparent in the low weight classes (Figs. 12 and 13). Among arboreal mammals, weight classes up to 150 grams seemed to be inadequately represented in the canopy samples when compared with that on the ground. However, these samples are a result of a trapping procedure which depends on an attraction to bait in traps on the part of the species present. When other methods are employed, such as searching for nests in arboreal tree cavities, a different estimate is derived of the mammals present in a given area (Fig. 14). In Malaysian forests a new assortment of species now appear, which were previously considered extremely rare (mainly as a result of trapping surveys). Yet, some of these species, such as *Hylopetes spadiceus* are extremely common. The ecological lesson to be learned from this is that in an epidemiological survey in which trapping is the only method for obtaining samples of potential host animals, a significant proportion of a mammalian population may be missed or numerically underestimated. Yet the predisposition of various species of mammals to involvement in transmission cycles of zoonotic pathogens is not necessarily correlated with, nor dependent on, their predisposition to enter traps.
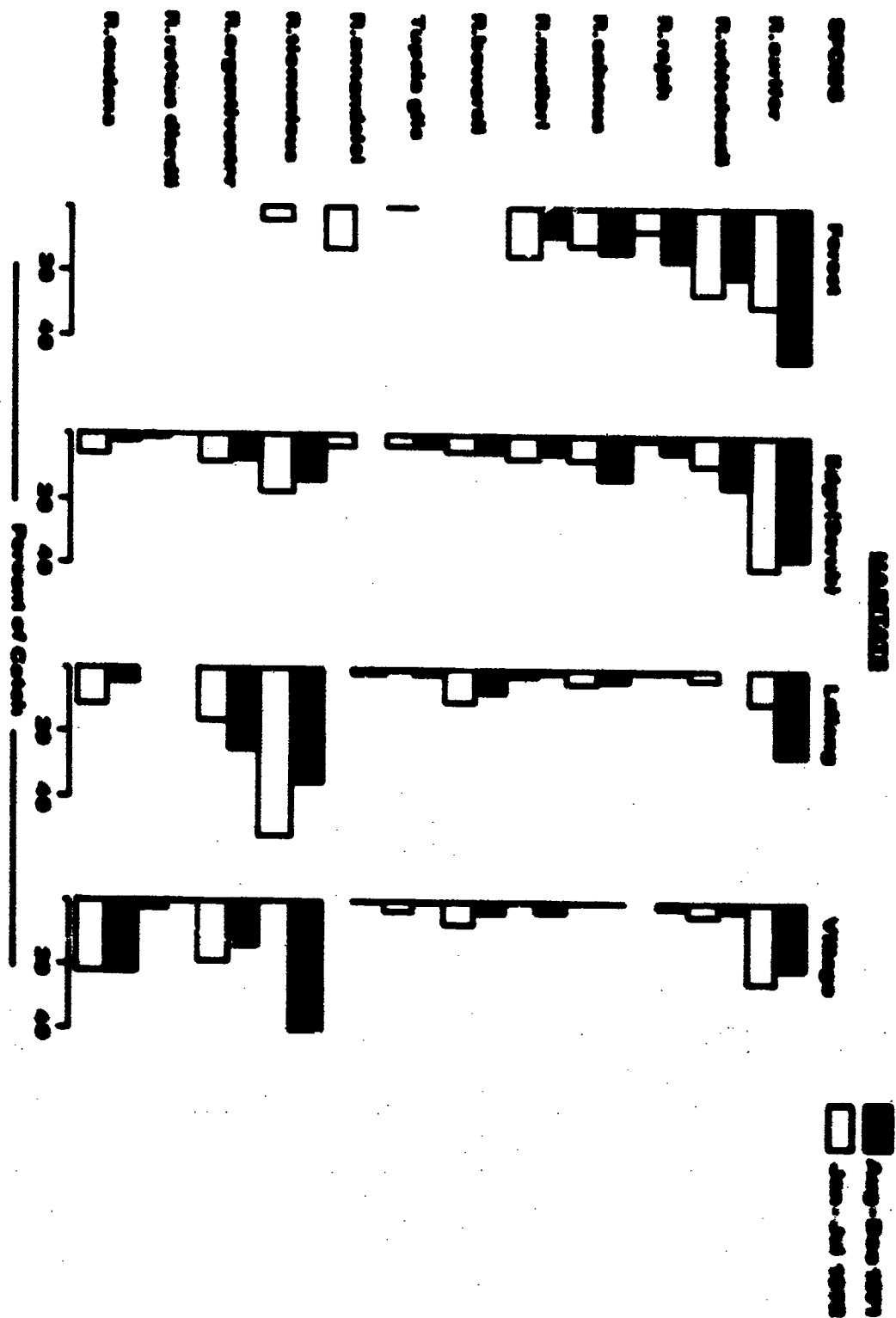
Figure 11
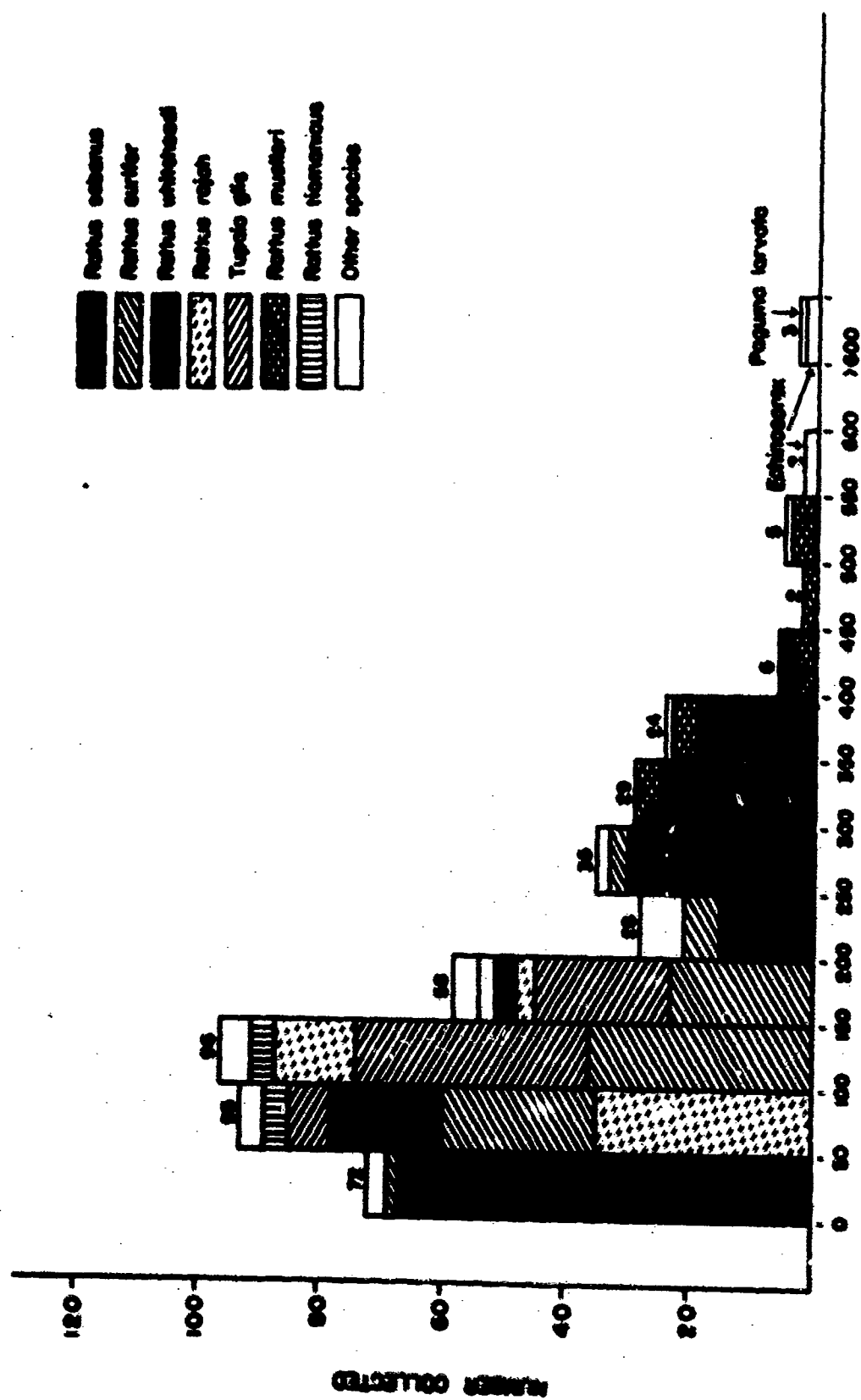
Figure 12

CANOPY (N=273)

NUMBER COLLECTED

WEIGHT CLASS (Grams)

Figure 13

Legend:
- Callosciurus nigrovittatus
- Callosciurus notatus
- Nycticebus coucang
- Sundasciurus tenuis
- Other species

NESTS IN TREE CAVITIES
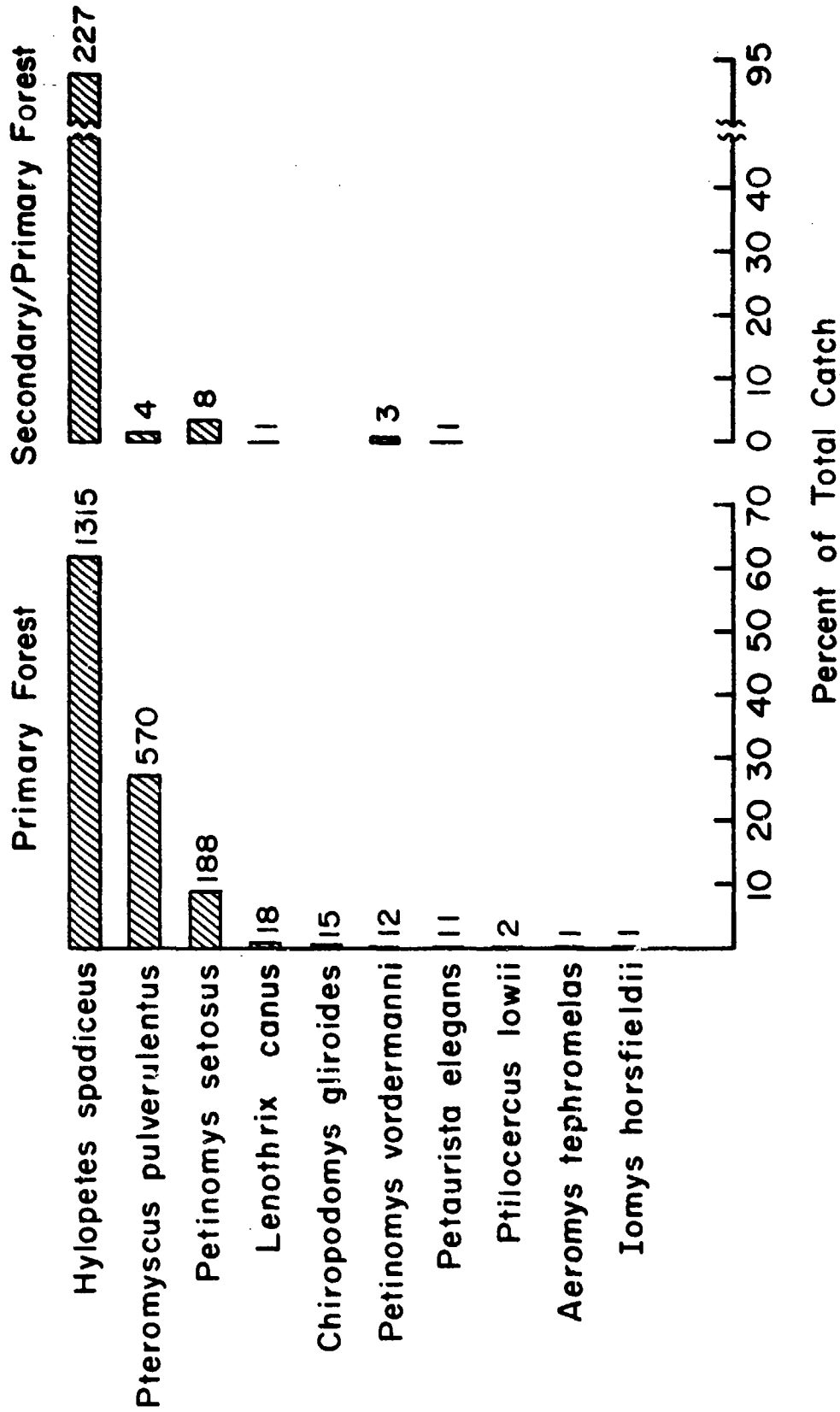
Figure 14

Another method of capturing animals employed in our studies was to search for nests in tree cavities and capture the inhabitants by hand. The dominant species, *Hylopetes spadiceus*, in the samples from tree cavities (Fig. 14) falls in the 100-150 gram weight class. The third most abundantly collected species usually weighs less than 100 grams. Smaller nocturnal, arboreal canopy species are also known to be present in the forests, but they have thus far eluded our attempts to capture them in significant numbers. Also of note is that *Lenothrix canus*, *Chiropodomys gliroides*, and *Ptilocercus lowii*, were caught in traps at a greater proportion of the total trap collections than in the total collections from tree cavities which they use for nests.

This nest collecting technique also demonstrates differences in habitats, in that *Pteromyscus* and *Petinomys setosus* occurred relatively less frequently in partially logged forest (secondary/primary) collections than in those from primary forests (Fig. 14). Thus, these species could serve as good indicators in surveys as to the characteristics of various types of forest. Other types of forests show markedly different species compositions both in the flora and fauna. For example, some casually cultivated rubber forests, with trees of various ages, interspersed with fruit trees, palms, and other species, yielded *Petinomys vordermanni* as the dominant species in dead tree cavities and *Iomys horsfieldii* in cavities in live trees (Muul and Lim, 1971).

Seasonal Periodicity and Cycles of Infection: The underlying factors that govern seasonal phenomena of biological events in evergreen tropical rainforests are still not apparent. The seasonality of scrub typhus infections in various types of habitats for 1969-1971 was already discussed above. These results are not clear as yet, but it appears that something other than a simple annual cycle is operant.

The data from the prospective linear studies begun in August 1971 of animals and scrub typhus in different habitats at Bukit Lanjan have been summarized through about February 1972 (see Table 1). The rates of isolation of rickettsia from indicator species averaged from 24 to 46 per cent or more. No marked seasonal patterns of isolation rates have emerged in these 6-7 months for most of these species, with the exception of the forest species, *R. sabanus*, which averaged between 27 and 48 per cent positives from September through November (N=15-21) to 7 and 17 per cent in December 1971 and January 1972 (N=14,12). By March the isolation rate was back up to 37 per cent (N=19). With the exception of *R. argentiventer* in lalang, the isolation rates in the forest were higher than in the other habitats. However, the latter showed no marked seasonality in isolation rates.

Seasonal Periodicity - General Considerations: McClure (1966) was one of the first to demonstrate variations in the seasonality of phenology of trees in Malaysia, showing that some had an annual pattern, but that others had several seasons per year and that some had seasons exceeding a year interval. Ecological data have been collected by the Department of Ecology to analyze these phenomena

over the last several years in conjunction with our other studies.
These include studies of seasonality of reproduction, food
utilization, parasitizations, etc. Patterns, are emerging including
unsuspected ecological phenomena, such as supra-annual cycles.
Summarizations of these data will be presented in the near future.
Their relationship to unexpected patterns in the temporal distributions
of prevalence of pathogens, once understood, should provide a measure
of predictive ability to recognize places and times of potential
hazard in terms of susceptibility of humans to exposure to infections.

Zoogeographic Studies of Mammals, Scrub Typhus and Other Pathogens:
The data on mammals collected in May 1971 in East Malaysia (Sabah) were
presented in preliminary form in last year's report (1971). The
results of FA tests of blood samples collected from these mammals are
now available (Tables 2-4). Although serological results cannot be
compared in a precise way to results of isolation attempts (Fig. 15
for W. Malaysian mammals shows the association between isolation
attempts and serological results), nevertheless, they provide a
measure of scrub typhus rickettsial activity in most mammals.

As can be seen in Table 2 the overall proportion of serologically
positive reactors was much lower than that observed through most of the
months in West Malaysia (see May 1971 in Fig. 10). Of course,
seasonal factors have not been accounted for in terms of comparisons
of these geographically separated areas. Nevertheless, as was the
case in West Malaysia, the arboreal species seemed to be much less
involved in the transmission cycle of scrub typhus rickettsiae than
were the semiarboreal and ground species (Table 3). Likewise, as was
the case in West Malaysia, the animals from edge habitats ("scrub")
had a lower rate of positive reactors than did those from forests
(Table 4). Significantly, those species caught in the edge habitat
that are characteristic of forest *(R. sabanus, R. muelleri)*
contributed disproportionately greatly to the numbers of positives
(Table 2).

The analyses of additional data, such as, for blood parasites
and other parasites, is not yet complete.

Data for mammals and results of serological tests for material
collected in Indonesia (Java) are given in the Acarology section of
this report.

Ecological Niches and Predisposition to Infections: Several
aspects of ecological niches have been discussed above that have been
demonstrated to be associated with predisposition of species to
involvement in disease transmission cycles. For example, the degree
to which the mammals are arboreal and their habitat associations
seemed to correlate with the prevalence of scrub typhus rickettsial
isolations from various species. The Annual Report for 1970
summarized data on *Plasmodium* infections which seemed to correlate
with daily temporal patterns of activity of hosts. More data which
support this have been collected. Still other observations remain to
be explained in relation to presence or absence of particular
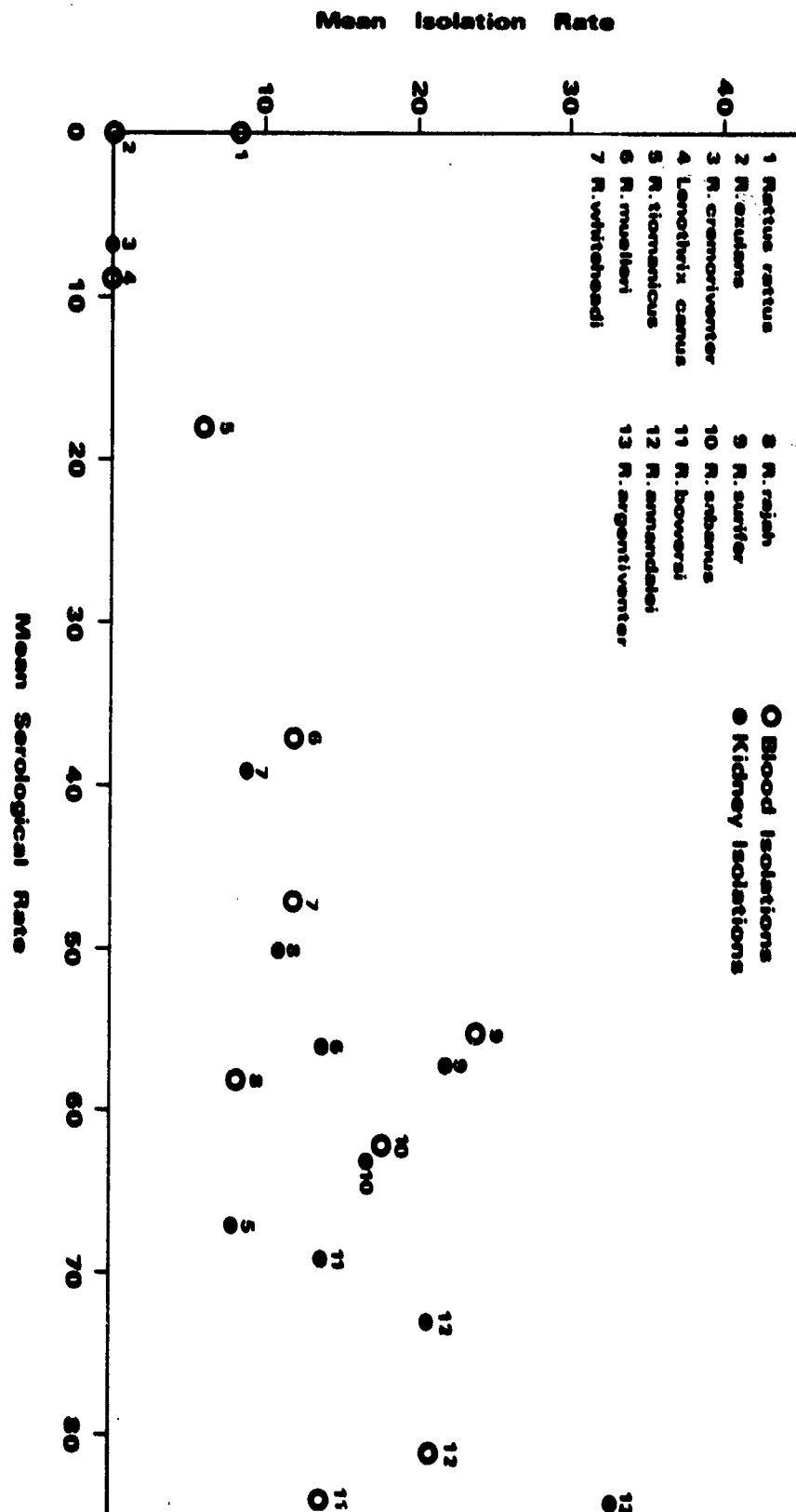
Figure 15 Relationship between mean isolation rate (blood and kidneys) from rats and the mean rate of serological positives.

Table 2

Numbers of ground and semiarboreal mammals captured in May 1971, tested and found to be serologically positive reactors in FA tests for scrub typhus *(Rickettsia tsutsugamushi)* antibodies. (East Malaysia: Sabah: near Ranau) (See Figure 10, for West Malaysia)

	No. Captured	No. Tested	No. Positive
R. cremoriventer	153	100	11 (11%)
Tupaia tana	100	86	0
R. muelleri	76	56	13 (23%)
R. whiteheadi	90	33	12 (36%)
R. sabanus	31	26	4 (15%)
R. surifer	11	10	0
R. rattus	8	5	0
R. tiomanicus	3	3	0
R. exulans	4	2	0
Tupaia glis	2	2	0
R. rajah	1	1	1
	479	324	41 (13%)

Table 3

Vertical zonation within the forest and scrub typhus
(*Rickettsia tsutsugamushi*) serological data for small
mammals in East Malaysia (near Ranau, Sabah).

	No. Tested	No. Positive	% Positive
Arboreal	141	2	2
Semiarboreal	224	15	7
Ground	100	26	26

Table 4

Habitat distribution and scrub typhus (Rickettsia tsutsugamushi) serological data concerning ground and semiarboreal mammals in East Malaysia (near Ranau, Sabah). Numbers collected and numbers tested appear in parentheses

	Primary Forest		Secondary Forest		Edge Habitat ("Scrub")		Village	
	% Species Catch	% Positive	% Species Catch	% Positive	% Species Catch	% Positive	% Species Catch	% Positive
R. cremoriventer	78 (120)	11 (72)	10 (15)	17 (12)	12 (18)	6 (16)	0	-
Tupaia tana	72 (72)	0 (62)	14 (14)	0 (10)	14 (14)	0 (14)	0	-
R. mulleri	71 (54)	28 (40)	19 (15)	11 (9)	10 (7)	14 (7)	0	-
R. whiteheadi	52 (47)	33 (18)	25 (23)	60 (10)	13 (12)	0 (4)	0	-
R. sabanus	74 (23)	17 (18)	16 (5)	0 (5)	10 (3)	33 (3)	0	-
R. surifer	65 (7)	0 (7)	0 (0)	-	35 (4)	-	0	-
R. rajah	0 (0)	-	0 (0)	-	- (1)	pos (1)	0	-
Tupaia glis	- (1)	-	- (1)	-	0	-	0	-
R. tiomanicus	0	-	- (1)	- (1)	- (2)	0 (2)	0	-
R. exulans	0	-	"50"(2)	0 (2)	"50"(2)	0 (2)	0	-
R. rattus	0	-	0 (0)	-	50 (4)	0 (4)	50 (4)	0
% of total catch	70 (324)	13 (217)	16 (76)	18 (49)	9 (67)	8 (49)	<1 (4)	0

pathogens in small mammals that can be grouped according to ecological factors. For example, among the mammals collected in East Malaysia the ground dwelling and semiarboreal mammals show a certain prevalence rate of serological positive reactors (Table 4). Yet, among these is a species, *Tupaia tana* (a tree shrew) which had no positive reactors among 86 samples tested. In West Malaysia, a closely related species (both systematically and ecologically), *Tupaia glis*, had a very high serological rate (over 70% positive reactors, see Table 22, Rickettsiology Section). Such parallel cases showing such vast differences in involvement(or apparent involvement) in disease transmission cycles point to lines of investigation which are likely to yield insight into the understanding of the complexities of rickettsial transmission cycles in nature, and ultimately add to our predictive ability to anticipate man's involvement under specific circumstances.

Systematic and Taxonomic Studies Related to Medical Ecology:
These studies are continuing. Aside from their inherent value to the understanding of the dynamics of mammalian distribution and ecology, they have aided medical studies. For example, the conjugates prepared for scrub typhus serological tests were made against groups of sera from species currently thought to be systematically related. In terms of immunology it would be expected that groups thus related would share more antigenic sites than groups assembled randomly or on the basis of only apparent relationships. These conjugates prepared as described have served us well in our surveys of scrub typhus activity in small mammals.

Specimens Examined: In addition to the species listed in last year's report, *Cynogale bennettii* (otter civet) has been added to our records. Additional specimens of extremely rare *Petaurillus kinlochii* have also been obtained.

Cooperative Studies: Dr. F. C. Colley and Dr. S. Mullin have continued to report new host records and new species of *Eimeria* and Coccidia, from material provided by the Division of Medical Ecology.

Lice collected by the Division of Medical Ecology are being studied by Dr. K. C. Emerson for taxonomic and distributional information.

Fleas collected by the Division of Medical Ecology are being studied by Dr. R. Traub for taxonomic and distributional information.

Ticks collected by the Division of Medical Ecology are being studied by Dr. H. Hoogstral for taxonomic and distributional information.

Detailed taxonomic studies of rats, beyond the scope of our protocols, are being carried out by Dr. G. G. Musser at the American Museum of Natural History in New York, to supplement the information available to us through our own studies.

Phenological studies are being carried out by Dr. Francis Ng of the Forest Research Institute, Kepong, Selangor to determine the seasonal patterns of botanical activity in forest under study by us.

Other cooperative studies are in progress with research institutes such as the Smithsonian Institute in Washington, D.C., and others in London, Leiden (The Netherlands) and elsewhere to aid us in data processing schemes, in identifications of materials, in providing comparative materials, in providing copies of difficult to get literature, in establishing international contacts for possible future studies, evaluations of specialized manuscripts etc.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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RESPONSIBLE INDIVIDUAL NAME: Dr. R. Bhagwan Singh, Acting Director TELEPHONE: Institute for Medical Research	PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: Walker, J.S., MAJ, VC TELEPHONE: SOCIAL SECURITY ACCOUNT NUMBER:
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23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) Technical Objectives: To continue to update the facilities and caging of the research and breeding colonies of laboratory animals, to study and develop procedures that can be used for maintaining the silvered leaf-monkey, to maintain and improve laboratory colonies of the wild rat, *Rattus annandalei*, and mouse deer.

24.(U) Approach: In order to update the laboratory animal colonies, new breeding stock will be obtained for the guinea pig colony and new caging for the rat and hamster colonies and a new facility designed; studies will be initiated beginning at the time of capture of silvered leaf-monkeys and run through one year of captivity. These will include normal values for this species. Detailed records will be kept on the mouse deer and *R. annandalei* colonies so that data can be obtained on their reproductive capacity in the laboratory.

25.(U) Progress: New breeding stock was obtained for the guinea colony from the U.S. and replacement is being carried out over a year's time. New caging was obtained for the rat and hamster colonies bringing them up to standard and a new animal facility was designed for the IMR complex. Studies were conducted and completed on the maintenance of the silvered leaf-monkey and normal values obtained. Detailed records were kept on the mouse deer and *R. annandalei* colonies and production capacity obtained.

Available to contractors upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

LABORATORY ANIMAL DEVELOPMENT AND ZOONOTIC DISEASES

Breeding Colonies

General: No problems were encountered during the last year with the mouse, rabbit, guinea pigs, hamster or rat colonies maintained and run jointly with the Institute for Medical Research. Both the mouse and guinea pig colonies were expanded. The rat and hamster colony were recaged and brought up to standard.

New breeding stock was obtained from WRAIR for the guinea pig colony. As offspring become available, this new stock will replace the local stock. It is anticipated that within the next year all local multicolored stock will be replaced with the Hartley albino strain.

New Facility: During this reporting period we were asked by the Institute for Medical Research to assist them in designing a new laboratory animal facility to house not only the breeding colonies but also all experimental animals located at the Institute. This was done in consultation with the Department of Veterinary Medicine, SEATO Medical Research Laboratory. The preliminary plans were turned over to the government architect and final drawings should be ready within a few weeks. It is anticipated that construction will begin around the first of October 1972 and it is scheduled for completion by December 1973.

The facility is designed so that the breeding and experimental colonies are completely separate in operation including cage washing and storage. It will contain approximately 50,000 square feet and be of open construction. The walls will be screened and wired panels. It will be cement and metal (nonferrous) construction and rodent proof. The clinical laboratory, surgery, office, inoculation rooms, and high risk animal rooms will be air-conditioned. There will be one self-contained high risk isolation suite with animal rooms, change rooms, autoclaves, and sterilization capacity for liquid effluent.

Silvered Leaf-Monkeys

General: During the last two reporting periods, preliminary reports were made on maintenance and disease problems in the laboratory and their susceptibility to scrub typhus. These studies have been greatly expanded and are reported under scrub typhus. In order to have sufficient animals, considerable effort was expended in refining and working out the colony problems of this species and obtaining normal values.

Capture, Handling and Survival

Because the silvered leaf-monkey does not have the curiosity of the macaques and is arboreal they cannot be caught in traps even by

the aborigines and must be captured either by the use of paralytic agents or by hand. The latter method was used for our animals. The method is to locate a troop in small mangrove trees, surround them and then shake them out to the ground. By this method as many as 25 monkeys can be captured within 4-5 hours.

The local exporter and biomedical investigators in Malaysia gave us little encouragement in that they said the silvered leaf-monkey was very "fragile" and wouldn't survive in the laboratory. To test the reasons, we arranged for the local exporter to obtain a large group of animals which were to be divided into two groups. One group was to be delivered to our laboratory within 72 hours and the other group was to be conditioned by him in his facilities. We received 30 animals and the local exporter kept 25. Table 1 gives the survival results in the two facilities. The group of 30 animals in our laboratory received extensive care. Table 2 gives the causes of death for the 18 animals dying in our laboratory. In general, the animals dying at the local exporters died of the same causes, the only difference being that a larger percentage died of diarrhea. Isolates of enteric pathogens were made from 5 of the 18 animals dying in our laboratory. They involved two genera, *Salmonella* and *Escherichia*. Three types of *Salmonella* were isolated, B, D and El while two types of pathogenic *E. coli*, 0111:B4 and 0119:B14 were isolated. One animal that died yielded all three types of *Salmonella*. Many of those dying from salmonellosis had an acute peritonitis due to perforations of the gastointestinal tract. A bacterin (10^8 organisms/ml) composed of the above isolates was prepared and used in subsequent groups of animals.

After a field trip to investigate facilities and methods from the time of capture, and our experience in the laboratory new procedures were instituted.

(1) Laboratory personnel are present at the time the animals are caught and immediately take charge of them.

(2) We provide cages and transportation back to the laboratory.

(3) In the field, our technicians do the following:

    (a) take fecal and blood specimens
    (b) give each monkey injections of procaine penicillin in oil, promazine hydrochloride, and the bacterin
    (c) mark each animal for future identification

(4) The technicians then place them in cages that are high enough (3 feet) to allow the monkeys to stand up and move about and immediately bring them to the laboratory. Upon arrival at the laboratory they are given food and water. On day three, they are given another injection of procaine penicillin in oil and 1 cc of B complex if they are exhibiting anorexia. On day seven they are tattooed, weighed, temperature determined and blood and fecal specimens taken. At the same time, another injection of penicillin and the bacterin is given. The bacterin is repeated again at 28 days.

Table 1

Survival Rates on the 1st Group of Silvered Leaf-monkeys
Captured

Facility	Number	Survival	
		Number	Percentage
Local Exporter	25	2	8
USAMR'	30	12	40
Totals	55	14	26

Table 2

Causes of Death in the 1st Group of 30 Silvered
Leaf-monkeys brought into the Laboratory

Cause of Death	Number	Percentage
Diarrhea	12	67
Exhaustion or shock*	2	11
Metahemoglobinemia**	2	11
Suspected viral***	2	11
	18	100

* Exhaustion: Arrival at the laboratory in a
state of exhaustion and dying
within five days of arrival.

** Metahemoglobinemia: Animals that died
suddenly on the same day that
the diagnosis of metahemoglobinemia
was made on one case that was
in a state of collapse.

*** Suspected Viral: Diagnosis made on the basis
of clinical impression and a
leukopenia.

The survival rates in the three groups handled as outlined above are given in Table 3.

Causes of Death

The causes of death fit into 7 etiologies some of which occurred in all the 4 groups of animals, see Table 4. The diarrhea problem seems to have been solved by bringing them directly into the laboratory, thus avoiding contamination at exporters primate holding areas. Also contributing to this decrease could be the use of the bacterin and washing all greens in chlorex (1 tsp/gallon) before feeding. The largest losses are due to exhaustion and shock, particularly in groups 2 and 4. In both of these groups, there was a break in technique in that the animals were caught the evening before we obtained them and were held without adequate food or water and in crowded conditions overnight.

Normal Values

Since little information is available on normal blood values for this species, we initiated a study to determine the values in the wild (at the time of capture) and how they changed in the laboratory. Table 5 gives the values on temperature, packed cell volume (PCV) and WBC. One surprise was that the variation in rectal temperature was so slight. The WBC levels did not change significantly during 9 weeks of conditioning. However, the PCV did change dramatically. One group of 33 animals was closely followed for changes in red blood cell count (RBC), hemoglobin (Hg) and serum protein levels (SP) over the first 8 weeks of conditioning. (Table 6) The RBC and Hg levels increased in parallel, RBC 41% increase and Hg 44% increase. The PCV increased 28% and the SP 14%. The erythrocyte indices are given in Table 7 for the same group of 33 animals. There was no change in either MCHC on MCH over the eight week period. The MCV did decrease slightly but because of the variability the decrease is not significant. Therefore the anemia that exists in the silvered leaf-monkey in the wild is a normocytic normochromic anemia.

The detailed picture of the white blood cell elements is given in Table 8. Twenty-four animals were bled 5 times over a 10 day period giving an N value of 120.

Serological Survey for Diseases Endemic to South East Asia

It was hypothesized that because the silvered leaf-monkey was arboreal, the habitat was mangrove swamp and it avoided all human contact, that it should not have come into contact with or have been exposed to several endemic diseases of the region. Serologic studies were performed not only on silvered leaf-monkeys but on the two ground dwelling macaque species of Malaysia, pig-tailed monkeys *(Macaca nemestrina)* and the crab-eating macaque *(Macaca fascicularis)* for comparison. (Table 9)

Table 3

Survival Rates of 3 Groups of 125 Silvered Leaf-monkeys

Group	Date of Capture	Total Number	Number Surviving	Survival Percentage
2	May 71	41	29	,1
3	Jul 71	35	22	63
4	Oct 71	49	33	67
	Totals	125	84	67

Table 4

Cause of Death in 4 Groups of 155 Silvered Leaf-monkeys

Etiology	Group Number	Number dying in each group	Totals	Percentage
Diarrhea	1	12	12	20
Exhaustion & shock	1	2		
	2	10		62
	3	8	36	
	4	16		
Liver abscess	4	1	1	2
Metahemoglobinemia	1	2	2	3
Pneumonia	3	2	2	3
Suspected "Viral"	1	2	6	10
	2	2		
	3	2		

Table 5

Normal Values for Temperature, Packed Cell Volume and Total White Blood Cell
Count in Silvered Leaf-monkeys

Time	No.	Temperature (F°)			PCV (%)			WBC (NO./ml. x 1000)		
		M	SEM	SD	M	SEM	SD	M	SEM	SD
Jungle	96	-	-	-	35	0.1	1.4	13.6	0.5	4.9
2 weeks	100	101.6	0.1	0.9	38	0.3	3.2	10.6	0.3	3.1
3 weeks	65	101.8	0.2	0.6	40	0.4	3.6	13.2	0.5	4.1
5-6 weeks	70	102.0	0.1	0.8	42	0.4	3.4	13.1	0.4	3.7
8-9 weeks	53	101.7	0.1	0.7	44	0.6	4.3	12.8	0.5	3.3

Note: M = Mean, SEM = Standard error of the mean, SD = Standard deviation

Table 6

Normal Values and Changes Following Capture in PCV, Red Blood Cell Count,
Hemoglobin and Serum Protein Levels for 33 Silvered Leaf-monkeys

Time	PCV (%)			RBC(No./cm^{3}x10^6)			Hg (gms%)			SP gms%		
	M	SEM	SD	M	SEM	SD	M	SEM	SD	M	SEM	SD
Jungle	36	0.5	2.7	4.1	0.2	1.1	-	-	-	6.1	0.2	0.2
1 week	36	0.4	2.5	4.5	0.2	1.0	8.2	0.1	0.7	6.0	0.1	0.6
2 weeks	38	0.6	3.8	4.9	0.1	0.7	8.9	0.2	1.0	6.3	0.1	0.5
3 weeks	42	0.4	2.5	4.4	0.1	0.8	8.7	0.2	1.2	6.5	0.1	0.6
4 weeks	42	0.5	2.6	5.2	0.2	1.0	9.1	0.3	1.5	6.5	0.1	0.6
5 weeks	42	0.5	3.1	5.1	0.2	1.1	9.9	0.3	1.5	6.7	0.1	0.6
6 weeks	44	0.4	2.4	5.7	0.2	0.9	9.7	0.2	1.4	6.8	0.1	0.7
8 weeks	46	0.5	2.8	5.8	0.2	0.9	11.3	0.2	0.8	6.9	0.1	0.6

Table 7

Erythrocyte Indices for 33 Silvered Leaf-monkeys

Time	Index MCHC (%)			MCH (ng.)			MCV (cu^3)		
	M	SEM	SD	M	SEM	SD	M	SEM	SD
Jungle	24	0.3	1.7	20	0.8	4	88	2	9
8 weeks	24	0.2	1.3	20	0.5	3	80	2	11

Mean cell hemoglobin concentration (MCHC) = $\dfrac{\text{Hemoglobin (Gm./100 ml)} \times 100}{\text{PCV}}$

Mean cell hemoglobin (MCH) = $\dfrac{\text{Hemoglobin (Gm./100 ml} \times 10}{\text{RBC count (million/cmm)}}$

Mean cell volume (MCV) = $\dfrac{\text{PCV} \times 10}{\text{RBC count (millions/cmm)}}$

Table 8

White Blood Cell Elements for 24 Silvered Leaf-monkeys

Cellular Element	%			Absolute Count		
	M	SEM	SD	M	SEM	SD
Basophiles	1	0.1	1.2	76	11	120
Eosinophiles	4	0.4	3.9	456	40	440
Monocytes	2	0.2	2.2	278	30	330
Myelocytes	2	0.2	2.2	211	10	110
Lymphocytes	39	1.1	12.0	4,126	151	1,650
Neutrophiles juveniles	1	0.1	1.1	59	10	105
bands	2	0.3	3.9	226	41	444
segments	49	1.3	13.7	5,143	182	1,992
WBC No./cmm x 1000	10.7	0.2	2.6			

Number of observations = 120 (24 animals x 5 samples over a
10 day period of time)

Table 9

Serologic Results to 6 Endemic Pathogens of Southeast Asia

Species	Salmonella Group		Shigella Group		Pathogenic *E.coli*		Melioidosis		*E. histolytica*		Scrub Typhus	
	N	% POS	N	% POS	N	% POS	N	% POS	N	% POS	N	% POS
Silvered Leaf-monkeys	79	>1	79	0	79	0	85	0	85	2	75	0
Pig-tailed Monkeys	-	-	-	-	-	-	95	5	-	-	75	36
Crab-eating Macaques	-	-	-	-	-	-	3C2	8	-	-	75	44

Antigens

Salmonella group = bacterial agglutination, positive titer 1:80
Shigella group = bacterial agglutination, positive titer 1:80
Pathogenic *E.coli* = bacterial agglutination, positive titer 1:80
Melioidosis = hemagglutination (HA), positive titer 1:10
E. histolytica = hemagglutination (HA), positive titer 1:64
Scrub typhus = indirect fluorescent antibody test, positive titer 1:40; trivalent antigen (Karp, Gilliam, Kato)

Serological Response to Salmonella Infection and the Enteropathogen Bacterin

The first group of 32 animals had an infection with several enteropathogens. Six of the survivors were checked to determine whether or not they had serologic conversion. Of the six animals, 2 became seropositive for Salmonella group antigen but none to the Shigella group antigen nor to pathogenic *E. coli*.

Serologic studies were done on 49 animals which had received the bacterin (10^8 organism/ml) composed of *Salmonella paratyphoid* B, Salmonella L and El, and pathogenic *E. coli*, 0111:B4. Of these, 32 or 65% converted to positive at 6 weeks for the Salmonella group antigen, only 8% converted to positive for the pathogenic *E. coli* antigen.

Summary

Presbytis cristatus can be maintained in a laboratory colony if procedures are adjusted to its habits, diet, and susceptibility to common human diseases. The latter, susceptibility to common human diseases, makes this monkey extremely valuable as a model for human diseases and has recently allowed significant advances to be made in the study of scrub typhus.

Their diet in the area from which we obtain them appears to be deficient in that they have a normocytic normachromic anemic which is correctable when they are fed a balanced monkey chow. Two possibilities for this deficiency are a protein deficiency (supported by the fact that they have hypoproteinemia in nature) and/or a copper deficiency (common in tropical areas such as Malaysia where the soils are leached by heavy rains).

Care immediately following capture and during the first weeks of conditioning is extremely important. Experience has shown that if they are handled as macaques are during this time, the mortality will approach 90+%. In fact, the primate exporters of the area say that silvered leaf-monkeys are extremely "fragile" meaning that they lose most of them. We have found that if they receive good care and handling starting at the time of capture and if sanitation levels are high, survival rates are good (67%). Most losses occur within one week of capture except in cases where they become exposed to human pathogens such as pathogenic *E. coli* and parathyroid fever. It is advisable to vaccinate the animals against these two organisms since they are endemic in the area and it is almost impossible not to have them exposed at one time or another. Losses after the first 2 months have not occurred in our laboratory. We now have seven animals that have been in the laboratory for over 1 year.

Mouse Deer

General: During the last two years this Unit has reported on the maintenance and breeding of the lesser mouse deer, *Tragulus*

javanicus in the laboratory. At first, the rate of success was marginal. However, last year a detailed report on their caging, diet and survival problems was made as well as preliminary work on normal blood values. During this reporting period, employing methods worked out previously considerable success was obtained both in survival and breeding.

Diet: The diet was simplified from that reported last year in that the sweet potatoes, green beans and fruit (apples and oranges) were dropped. The current diet, used now for 9 months, consists only of rabbit and guinea pig chow plus *kangkong* (similar to spinach). In addition a multivitamin and lysine preparation is added to their drinking water.

Survival Rates: It was reported last year that it appeared that some of the animals went into a stress-shock (which generally results in hypoglycemia and/or hypoadrenocorticisin in ruminants) and preliminary data were presented on the results of prophylactic therapy. The study was continued this year, however, no animals were added to the untreated group controls since animals were urgently needed for malaria studies. The results are presented in Table 10. We doubt that any of the treatment combinations had statistically significant effect. The untreated controls were all early in the study and we found that the technicians were initially selecting the healthier animals for the untreated group. This was stopped and subsequently treatment was administered to every third animal regardless of condition upon arrival at the laboratory. What obviously made the most significant difference over the survival rates early in the study (22% for 67 animals) was the changes in caging, diet and husbandry. Also a significant factor in survival is the length of time between capture and arrival at the laboratory, see Table 10. We have found this to be true of the silvered leaf-monkey also. The animals must be carefully handled following capture and immediately brought into the laboratory or survival rates are very poor.

Breeding: Preliminary results were reported last year on the successful breeding of the mouse deer in the laboratory. During this reporting period 6 groups of animals were set up as breeders, (Table 11). All offspring reported were conceived in the laboratory colony and all mothers were wild caught. Two groups produced two and one group three offspring during this reporting period. The intervals between offspring were 113 days (3.8 months), 139 days (4.6 months), 143 days (4.8 months) and 277 days (9.2 months). Two offspring died, both within 3 days of birth, probably due to lack of proper nursing or lactation. Four of the offspring are now between 9 and 12 months of age. There were several young born to mothers within a couple months of capture, however, most of them died soon after birth. The reason again is unknown, however, it takes several months for the adults to be completely used to laboratory and most likely the mothers failed to produce sufficient milk.

Table 10

Survival of Wild Caught Mouse Deer and the Effect of Prophylactic Therapy

Time between Capture and Arrival at the Laboratory	Untreated	Therapy CG*	GG+P	P	Total
Within 24 hours	8/10 (80%)	16/25 (64%)	15/24 (63%)	15/18 (83%)	54/78 (70%)
Between 2 and 5 days	-	0/5 (0%)	1/5 (20%)	1/5 (20%)	2/15 (13%)

*Note: CG = calcium gluconate (10%, 10cc, IP)

GG+P = calcium gluconate plus prednisolone acetate

P = prednisolone acetate (25 mg, subcutaneous)

All of the above drugs were administered within 24 hours of arrival at the laboratory.

Table 11

Production of Mouse Deer Selected as Breeders in a Laboratory Colony over a 18 Month Period

Cage No.	Parents		No. of Offspring (No. multi-births)	No. of Survivers
	No. of Males	No. of Females		
1	1	1	3	3
2	1	1	1	1
3	1	2	2	1
4	1	1	2	2
5	1	1	2	1
6	1	2	2	2
Total			12	10

Rattus annandalei

General: Last year we reported preliminary results on the colonization of the wild rat *Rattus annandalei*. During this reporting period, in cooperation with the Department of Medical Ecology, an intensive study of their reproduction was started.

Breeding: Eight pairs of breeders were set up as they became available. All were captured animals from near Kuala Lumpur. Results are given in Table 12. The litter size ranged from 1 to 7, the average was 4.2. In all cases the male was left with the female and this often resulted in immediate rebreeding and reduced the interval between litters to as low as 22 days. There were several cases in which the interval was below 30 days. The litter size was affected by the number of previous litters which the female had had. The first litter was the smallest. Litter size increased for the first several litters and then decreased at the 4th and 5th litters. We recommend that they not be kept for more than 5 litters. Of the 112 offspring, only 2 died, which is very low for wild caught mothers. We now are setting up several F-1 breeding pairs and except production to increase.

Growth: Initially problems were encountered in obtaining weights at birth and during development. This was because the mother tended to kill the offspring when they were replaced in the nest. Once this was overcome by using a pair of clean sterilized long handled forceps to handle the young growth weights were obtained at various intervals, see Figure 1 The ranges of the weights are plotted in comparison to the means every eight days. One factor which greatly influenced the range during the first 3 weeks was litter size. Individuals from larger litters were much smaller than if only one or two offspring were produced. Growth during the first two weeks was much slower than after this period. Their eyes open between 10 and 16 days, mean 14, and by 3 weeks they are active in the cage and eating. We currently are weaning them at 28 days and it is felt that this could be cut to 21 days.

Use as Laboratory Animals: They react to experimentation and handling very well. During this reporting period, over 180 have been used in experiments, some of which lasted for 9 months. Many of them were caught and handled as often as twice a week during this period. One group underwent surgery twice in 9 months, first for removal of the spleen and the second time for removal of one kidney. Only two animals died out of more than 40 operated on. The experimental animals were housed in a standard wire bottom, hanging, rat cage such as is used for the common laboratory rats. The only modification was that a movable hinged door was placed halfway back in the cage and the sides of the rear half were solid metal. This was because it was observed that they dislike light and tend to hide during the day if given the opportunity. They tend to be nervous and bite and thus must be handled with a medium weight pair of gloves, however, because of their small size (mean adult weight 330 gms) this biting causes no real problems.

Table 12

Production of *Rattus annandalei* in a Laboratory Colony

Pair No.	No. of Litters	Average Litter Size (range)	Average Interval Between Litters, Days (range)	Total Production (over how long, months)
1	4	4.3 (4-5)	34 (29-38)	17 (4.5 months)
2	3	4.7 (4-5)	65 (31-100)	14 (4.2 months)
3	3	4.7 (3-6)	33 (29-37)	14 (4.2 months)
4	3	2.7 (2-4)	47 (40-54)	8 (3.1 months)
5	5	3.2 (1-6)	35 (26-62)	16 (4.7 months)
6	3	6 (5-7)	48 (42-54)	18 (3.2 months)
7	4	4.3 (2-6)	30 (22-35)	17 (3.0 months)
8	2	4.0 (2-6)	70	8 (2.3 months)
Total	27	4.2 (1-7)	41.5 (22-100)	112

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY 30 06 72	REPORT CONTROL SYMBOL DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISB'N INSTR'N	9A. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
30 06 71		U		N/A	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY		3A062110A831		
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

Investigations on Liver In Vitro

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
10 71	9 72		

17. CONTRACT/GRANT DADA17-72-G-9350

			18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (in thousands)
a. DATES/EFFECTIVE: 10 71	EXPIRATION: 10 72		PRECEDING		
b. NUMBER:		FISCAL	72	1.0	11.0
c. TYPE: Y Grant	d. AMOUNT: 218	YEAR	CURRENT		
e. KIND OF AWARD:	f. CUM. AMT.		73	1.0	15.4

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
NAME: US Army Medical Research Unit ADDRESS: Institute for Medical Research Kuala Lumpur, Malaysia	NAME: Institute for Medical Research ADDRESS: Kuala Lumpur, Malaysia
RESPONSIBLE INDIVIDUAL NAME: Dr. R. Bhagwan Singh, Acting Director TELEPHONE: Institute for Medical Research	PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: Cadigan, F.C., Jr., COL, MC TELEPHONE: SOCIAL SECURITY ACCOUNT NUMBER:
21. GENERAL USE	ASSOCIATE INVESTIGATORS NAME: Kyser, K.A., MAJ, MC NAME: Dondero, T.J., Jr., MAJ, MC

22. KEYWORDS (Precede EACH with Security Classification Code)

Liver trauma, liver regeneration, malaria, in vitro

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) Technical Objective: To attempt to culture of normal liver tissue for study of (1) factors affecting regeneration of liver, (2) certain biochemical activities of liver in health and disease, (3) exoerythrocytic stage of malarial parasites, (4) other intrahepatic infectious diseases.

24.(U) Approach: Tiny explants from liver removed from macaques at surgery were incubated in tissue culture medium containing homologous serum. Attempts were made at histological preparation of the tissues for observation. Radio-chemical techniques were used to assess hepatic parenchymal function. Various experimental conditions such as use of collagen in the tubes, temperature, and moving versus static conditions were investigated.

25.(U) Progress: The systems of liver explants, tissue culture medium and serum collected under various conditions do incorporate radioactive labelled biochemical substrates. In the presence of certain sera, particularly serum collected following partial hepatectomy and sera which were not treated to 56° for 30 minutes, incorporation of radioactive label was greater.

INVESTIGATIONS ON LIVER IN VITRO

Investigations on Normal Liver Tissue *In vitro*

Attempts were made to maintain explants of normal liver *in vitro* in order to study factors which promote liver regeneration and also to attempt to grow the primary exoerythrocytic stage of malarial parasites for possible studies of growth requirements of and drug action on this important stage.

Liver from pig-tailed macaques *(Macaca nemestrina)* was collected at surgery, cut into $1\frac{1}{2}$ mm cubes and placed in collagen lined tubes containing tissue culture medium M 199 (Grand Island Biologicals) and homologous serum and maintained at 39°C. In general, 4 pieces of tissue were cultured per tube. Medium and serum were changed every third day. Biochemical monitors of liver parenchymal functions – production and storage of glycogen, production and excretion of cholesterol and albumin – were adopted in order to evaluate stimulation or inhibition of the liver. At various times of incubation C-14 labelled glucose or sodium acetate was added to the medium and determination was made of the amount of isotope incorporated after 2 days into the tissue glycogen (Pfluger method: tissue digested in 30% KOH and glycogen precipitated in 60% ethanol), excreted cholesterol (chloroform extract of the medium), and albumin (TCA precipitate of the medium). Preliminary experiments indicated that the radioactive label was contained in the albumin fraction of the TCA precipitate. Technical problems were encountered in histological preparation possibly due to the friability of the infarcted cores of the liver pieces.

When isotope labelled substrates were added to the incubation mixtures of explanted liver, M 199 and serum the label was reproducibly incorporated into the three fractions. The times of incubation prior to pulsing with isotope which have been tested thus far are 1 day, 4 days, 8 days, 12 days, 16 days, 20 days, 24 days, 28 days. Longer studies are in progress. Serum collected after partial hepatectomy appeared to promote greater incorporation of isotope into the three fractions than normal, pre-hepatectomy serum. In general serum collected at 2 and 3 weeks following surgery seemed to stimulate incorporation of label into all fractions. 2-week post-hepatectomy serum was adopted as the routine serum additive for subsequent studies. Heat "inactivation" of both pre and post hepatectomy sera at 56°C for 30 minutes caused a reduction of isotope incorporation into the three fractions. In preliminary experiments, incorporation of label appeared comparable whether or not collagen was used in the tubes and whether continuous rolling or static conditions were used. Continuous rolling, however, was the routine method for incubation.

Because fairly high background-counts in all three fractions was encountered when heat killed liver was used, and in the chloroform extract and TCA precipitate fractions when no liver tissue was added, further work is under way to confirm and clarify the earlier findings.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION		DATE OF SUMMARY 30 06 72		REPORT CONTROL SYMBOL DD-DR&E(AR)4.16	
2. DATE PREV SUM'RY 30 06 71	4. KIND OF SUMMARY	5. SUMMARY SCTY U	6. WORK SECURITY	7. REGRADING N/A	8a. DISB'N INSTR'N NL	9a. SPECIFIC DATA-CONTRACTOR ACCESS ☑ YES ☐ NO		9. LEVEL OF SUM A. WORK UNIT-	
10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER		WORK UNIT NUMBER			
a. PRIMARY		3A062110A831							
b. CONTRIBUTING									
c. CONTRIBUTING									

11. TITLE (Precede with Security Classification Code)

Investigations of Malaria

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE 10 71	14. ESTIMATED COMPLETION DATE 9 72	15. FUNDING AGENCY		16. PERFORMANCE METHOD

17. CONTRACT/GRANT DADA17-72-G-9350		18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (in thousands)
a. DATES/EFFECTIVE: 10 71	EXPIRATION: 10 72	FISCAL YEAR PRECEDING 72	1.0	19.3
b. NUMBER:				
c. TYPE: Y Grant	d. AMOUNT: 218	CURRENT 73	1.0	20.1
e. KIND OF AWARD:	f. CUM. AMT.			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
NAME: US Army Medical Research Unit ADDRESS: Institute for Medical Research Kuala Lumpur, Malaysia	NAME: Institute for Medical Research ADDRESS: Kuala Lumpur, Malaysia
RESPONSIBLE INDIVIDUAL NAME: Dr. R. Bhagwan Singh, Acting Director TELEPHONE: Institute for Medical Research	PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME: Andre, R.G., CPT, MSC TELEPHONE: SOCIAL SECURITY ACCOUNT NUMBER:
21. GENERAL USE	ASSOCIATE INVESTIGATORS Cadigan, F.C., Jr., COL, MC NAME: Bolton, M., M.D., M.T.M.&H. NAME: Kyser, K.A., MAJ, MC Dondero, T.J., Jr., MAJ, MC

22. KEYWORDS (Precede EACH with Security Classification Code)
See Continuation Sheet

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) Technical Objective: To characterize the level of resistant *P. falciparum* malaria in Malaysia using *in vivo* and *in vitro* techniques, to determine the epidemiological factors involved in the gradient of resistance between Thailand and Malaysia, to study the vector species of malaria in certain parts of Malaysia, to compare the prevalence of malaria in deep jungle dwellers versus those residing on the jungle fringe, to observe the effect of residual spraying and drug prophylaxis in certain jungle areas, to study the mosquito fauna found in the jungle canopy, to study the life cycle of *P. traguli* using light and electron microscopy, to examine the erythrocytic, exoerythrocytic, and sporogonic stages of *P. youngi*, and to establish and maintain certain colonies of anophelines.

To attempt to reproduce the primary exoerythrocytic stage of malaria *in vitro* or a model for possible study of growth requirements and drug therapy. To modify and amplify existing culture techniques of the blood stage of *Plasmodium falciparum* for testing drug resistance *in vitro*.

24.(U) Approach: *P. falciparum* infections will be studied using certain *in vivo* and *in vitro* techniques to determine the level of drug resistance. Mosquitoes will be collected by using light trap collections, human bait trap collections larval and human biting collections. Dissections will be made of mosquitoes belonging to the *Anopheles* and *Monsonia* genera. Prevalence surveys will be made periodically among jungle dwellers living in deep jungle or on the jungle fringe. Electron and light microscopy techniques will be used to study certain animals malarias. Mosquitoes will be reared for experimental transmission studies.

Available to contractors upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 MAR 68 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

DD Form 1498, Research and Technology Work Unit Summary,
Item 24 Continued:

Infective malarial sporozoites were added to tissue cultures of normal
primate liver and after various periods of incubation the tissues
were inoculated into an uninfected recipient animal whose blood was
thereafter screened for patent malarial infection.

25(U) Progress: In Trengganu 3300 adult mosquitoes were collected,
but only 14 *Anopheles maculatus* were caught. In Kelantan 8800 adults
in October and 4000 in March were captured, with 7% being *Anopheles
spp.* in October and 22% in March. No *A. balabacensis* were found in
either the adult or larval collections. (small numbers were collected
during previous year.) A breakdown in drug prophylaxis and residual
spraying led to an increase in malaria in one of the indicator
villages located in deep jungle. Twenty-one people were positive on
Day 0, and none were positive on Day 7 following treatment with 25 mg.
chloroquine per kg. of body weight over a three day period. With
renewed supervision of the preventive measures, the malaria prevalence
among these people came down from 18% to 8%. In the fringe areas the
measures are apparently having some effect, although *A. maculatus* is
still caught attempting to feed inside sprayed houses. At Bukit
Lanjan 3400 mosquitoes were caught in light traps and 3100 during
human biting collections. Only 90 anophelines were captured and only
12% of these were caught at ground level. Overall, 63% of the adults
were found at ground level. Ten species of larval mosquitoes were
found in black glasses or bamboo cups at this study site. *Aedes
albopictus* was found breeding both in the canopy and at ground level.
40% of the anophelines were parous and 28% of the culicines, 7
positives were found. During studies of tragulid malaria, a new
species of Plasmodium was found. The new parasite measures about 7 μ
x 9 μ, whereas, *P. traguli* is only about 3 u. in diameter. Out of 84
hosts examined, 64 had *P. traguli* and 12 had *P. n. sp.* Many strains
were made of the various life stages of *P. traguli* and *P. youngi.*
The *P. youngi* strain may be inherently resistant to chloroquine. To
support transmission experiments a colony of *A. letifer* has been
established and maintained.

In four completed experiments one recipient became patent, one showed
a few parasites, and two remained negative. The modified techniques
for *in vitro* blood culture of *P. falciparum* for drug testing are in
the preliminary stages.

Keywords: *Anopheles*, canopy mosquitoes, chloroquine resistance,
culicine, malaria survey, Plasmodium, Tragulid malaria, West Malaysia,
malaria, liver stage of malaria, drug resistance, *in vitro*,
experimental infection, parasitology.

INVESTIGATIONS OF MALARIA

Research in malaria was conducted by the Department of Entomology and the Department of Parasitology. Certain aspects of the studies were carried out in collaboration with the Hooper Foundation, the Gombak Aborigine Hospital, and the Malaria Division of the Institute for Medical Research.

Basic investigations on chloroquine resistance in West Malaysia and the associated vectors found at these study sites were continued during this reporting period. Studies of malaria in jungle dwelling people and the effect of residual spraying and prophylaxis on the prevalence of malaria in fringe and deep jungle have been continued. Preliminary ecological and entomological research on the mosquito fauna of the jungle canopy has been concluded. Certain species of adult mosquitoes along with their associated larval and pupal skins have been added to the USAMRU reference collection. Intensive studies on tragulid (mouse deer) malaria have been carried out during the year. Studies of *Plasmodium youngi* were initiated, and a *P. cynomolgi* strain was started to support research on the exoerythrocytic stages *in vitro*.

Specific Objectives

1. Chloroquine-resistant Malaria:

(a) To characterize the level *P. falciparum* resistance by *in vivo* and *in vitro* methods.

(b) To evaluate what appears to be a gradient in the amount of falciparum resistance found across the Kra Isthmus that coincides with the political boundary between Thailand and Malaysia.

(c) To determine the principal vectors of malaria found within the study areas and the infection ratio of the anopheline mosquitoes.

2. Malaria in Jungle-dwelling People:

(a) To compare the prevalence of malaria in deep jungle dwellers with those residing on the jungle fringe.

(b) To study cases of falciparum malaria for possible chloroquine resistance.

(c) To observe the effect of residual spraying and drug prophylaxis in certain jungle areas.

(d) To determine the anopheline vectors in the jungle fringe and in deep jungle.

3. Studies on Jungle Canopy Mosquitoes:

(a) To determine the prevalence of various anophelines and culicines at canopy level and at ground level.

(b) To study zonation of the mosquito fauna within the jungle canopy.

(c) To determine oviposition sites and frequency of oviposition of various mosquito species at canopy level.

(d) To determine the infection ratio in certain mosquito species found within or below the canopy.

4. Studies of Tragulid Malaria:

(a) To study the erythrocytic stages of *Plasmodium traguli* and a new plasmodium species *(P. n.sp.)* using light and electron microscopy.

(b) To determine the exoerythrocytic cycle of *P. traguli* using light and electron microscopy.

(c) To study the sporogonic stages of *P. traguli*.

(d) To conduct transmission studies with *P. traguli* and *P. n.sp.*

5. Studies on Primate Malarias:

(a) To establish and maintain *Plasmodium cynomolgi* for sporozoite inoculation into liver tissue culture.

(b) To study the erythrocytic, exoerythrocytic, and sporogonic stages of *Plasmodium youngi* using both light and electron microscopy.

6. Mosquito Colonies:

(a) To establish and maintain various anophelines and culicines in an insectary to support transmission experiments.

(b) To study the life cycle of some colonized jungle-breeding anophelines.

7. Reference Collection:

To expand the reference collection of mosquito specimens to support local taxonomic studies.

Chloroquine-resistant Malaria

General Background: Studies on chloroquine-resistant falciparum malaria in West Malaysia were started in March 1969 (see Annual Reports dated 1968, 1969, and 1970). A formal publication of the results of this work is in the *Transactions of the Royal Society of Tropical Medicine and Hygiene,* Vol.66, No.4, 1972. During the past year an *in vivo* survey of jungle dwelling people at Pos Shean, Pahang, was conducted. Associated vector studies were concluded at the study sites in Trengganu, Perak, Kelantan, and Johore. An apparent gradient between a high amount of chloroquine resistance in Thailand and a low amount in Malaysia was found. It is proposed that future work be on the determination of what epidemiological factors account for this gradient. Correlation of *in vivo* results to those found *in vitro* in Malaysia and Thailand should help elucidate this problem from a parasitological viewpoint. Vector studies at other points along the Thai/Malaysia border could prove very valuable.

Trengganu Studies: The survey sites are located about thirty miles southwest of Kuala Brang. (See Map). Bordering the study area on the south is the Trengganu River and on the north the Telemong River. Immediately adjacent to the area on the west are the foothills of a main mountain range and Taman Negara (National Park). Small individual holdings of agricultural land are found throughout the area. Logging is carried out to a great extent in the secondary and primary forest areas. Monsoon rains tend to isolate all the study sites in the months of October through January.

Collections for mosquitoes were made on 3-8 October 1971 and 19-25 June 1972. The types of collection made in the October survey were human bait trap (HBT) collections indoor and outdoor bare leg collections, bare leg collections on the jungle fringe and larval collections. In June indoor and outdoor collections, bare leg collections and larval collections were made in areas where logging is being conducted. Two HBT sites were used each night from 1830-0800 hours. Two collectors operated each human bait trap. Mosquitoes were collected off the inside of the net every $1\frac{1}{2}$ hours. At the indoor-outdoor collection sites, one man collected all mosquitoes coming indoors to feed on him, and one man sat outside the house collecting those mosquitoes attempting to feed on him. Human biting collections on the jungle fringe were done by three men at each village. Bare leg collections in the logging areas were done by five men. Bare leg collections are conducted from 1830 to 2400 hours each night. Larval surveys were made at all the four villages, with emphasis placed on potential *Anopheles b. balabacensis* breeding sites. Locations of the survey sites and overall study area are shown in the 1969 Annual Report.

Results: During the October collection almost 3300 adult mosquitoes were collected (see Table 1). Of this 498 were *Anopheles spp.* or 15%. Only 14 *A. maculatus* were caught, primarily in human bait traps; one was found positive for oocysts. All of the anophelines found in larval collections were *A. barbirostris, A. kochi,* and *A. letifer.* No. *A. b. balabacensis* were found in either adult or larval collections. This species was collected in small numbers during previous collections. *Mansonia dives* and *Culex tritaeniorhynchus* were the main culicines captured in the adult survey.

Kelantan Studies: The study area is in Kelantan near the Thai/ Malaysian border. (See Map). The hospital clinic at Ayer Lanas was utilized throughout the study. The state capital, Kota Bharu, is fifty-six miles to the northeast. The population is mostly Malay with agricultural products, such as rice, rubber, maize and tobacco being theim main source of income. Forested mountains border the area on the south and the west. Logging is being done in the area.

Mosquito studies in the Ayer Lanas area were conducted at four villages--Gemang, Jakar, Jeli, and Nibong. Location of these study sites are shown in last year's annual report. Two surveys were done during this reporting period, one in October 1971 and the other in March 1972. Both surveys utilized the following collection techniques: CDC light traps (with solid CO_2), human biting collections, indoor-outdoor collections, and larval collections. Four nights were spent at each village. In the human biting collections, five men placed themselves on the perimeter of the village to catch mosquitoes attempting to feed on them. Also at the villages one man collected inside a house while another collected just outside. Both the human biting collections and the indoor-outdoor biting collections were made from 1830 to 2400 hours each night. Ten light traps were placed in each village 5-6 feet above the ground or 20-30 feet about the ground in the lower canopy. Each trap was baited with 1lb. of solid CO_2 which was placed in a styrofoam-insulated plywood box. A rubber tube carried the CO_2 gas from the box to a point 2-3 inches from the intake portion of the trap. Traps were turned on at 1800 hours and turned off at 0700 hours.

Mosquito larvae were placed in 70% ethanol or in their own breeding water in plastic containers. Those in alcohol were labelled for later processing and identification; those in water were reared out for their associated skins. Some anophelines were brought back to the insectary for colony purposes.

Results: Over 8800 adult mosquitoes were caught in October and about 4000 in March. Kelantan was in the midst of a drought in March. Table 2 summarizes the results of the October collections, and Table 3 shows the results of the March survey. Of the 8843 mosquitoes captured in October, 574 were anophelines. Twelve of these were *A. maculatus*. The two species most prevalent were *A. karwari* and *A. kochi.*

THAILAND

Scale Of Miles
10 5 0 10 20 30 40 50 60 70

N

7

3

PERAK KELANTAN TRENGGANU

9

1

6

WEST MALAYSIA

PAHANG

4 5

SELANGOR

2

8

REFERENCE
State Boundaries -------
International Boundaries ———

1. Kuala Brang   6. Woh Poo
2. Bukit Lanjan   7. Ayer Lanas
3. Fort Kemar   8. Ampang Reservoir
4. Pos. Shoan   9. Pos. Legap
5. Selek

SINGAPORE

Table 1

Summary of Mosquito Collection Results at Trengganu Study Site
3-8 October 1971

Species Collected	Method of Collection				Total
	Human Bait Trap	Bare Leg Collection	B.L.C.		
			Indoors	Outdoors	
Anopheles aconitus	5	5	1	5	16
Anopheles barbirostris	15	12	-	7	84
Anopheles crawfordi	-	1	-	-	1
Anopheles indiensis	23	56	3	18	100
Anopheles karwari	75	63	7	22	167
Anopheles kochi	48	48	-	38	134
Anopheles letifer	-	4	2	2	8
Anopheles maculatus	11	2	-	1	14
Anopheles tessellatus	3	7	-	1	11
Anopheles vagus	5	1	-	7	13
Aedes spp. (6)*	54	25	7	7	93
Armigeres sp. (1)*	2	1	1	2	6
Culex spp. (4)*	751	321	62	212	1346
Mansonia spp. (3)*	334	478	118	414	1344
Total	1326	1024	201	736	3287

* Number of different species

Table 2

Summary of Mosquito Collection Results at Kelantan Study Site,
23 October to 1 November 1971

Species Collected	Method of Collection					Total
	Bare Leg Collection	B.L.C. Indoor	Outdoor	Light Trap(W.CO$_2$) Ground	Canopy	
Anopheles aconitus	13	2	9	38	29	51
Anopheles barbirostris	6	1	10	6	18	41
Anopheles indiensis	-	--	3	3	9	15
Anopheles karwari	42	4	36	69	54	205
Anopheles kochi	24	4	3	77	85	193
Anopheles maculatus	10	1	-	-	1	12
Anopheles phillipinensis	3	-	-	1	2	6
Anopheles tessellatus	-	-	-	-	11	11
Acdes spp. (4)*	38	10	3	33	25	109
Aedomyia sp. (1)*	-	-	-	27	27	54
Armigeres sp. (1)*	10	5	10	30	33	88
Culex spp. (7)*	1088	398	1074	2027	2008	6595
Mansonia spp. (4)*	433	192	410	176	212	1423
Total	1667	617	1558	2487	2514	8843

* Number of different species

Table 3

Summary of Mosquito Collection Results at Kelantan Study Site,
11-18 March 1972

Species Collected	Method of Collection					Total
	Bare Leg Collection	B.L.C.		Light Trap($W.CO_2$)		
		Indoor	Outdoor	Ground	Canopy	
Anopheles aconitus	51	8	20	146	109	334
Anopheles barbirostris	11	3	5	7	6	32
Anopheles crawfordi	1	1	1	1	-	4
Anopheles indiensis	39	21	29	41	28	158
Anopheles karwari	7	-	7	2	2	18
Anopheles kochi	12	-	8	44	24	88
Anopheles maculatus	4	2	-	-	-	6
Anopheles peditaeniatus	3	1	6	-	-	10
Anopheles philippinensis	26	7	12	63	53	161
Anopheles tessellatus	12	5	22	16	6	61
Aedes spp. (4)*	22	3	13	3	-	41
Aedomyia sp. (1)*	1	-	-	2	-	3
Armigeres spp. (3)*	6	1	4	5	1	17
Culex spp. (7)*	788	199	381	504	469	2341
Mansonia spp. (3)*	305	117	154	73	66	715
Total	1288	368	662	907	764	3989

* Number of different species

872 anophelines out of 3989 mosquitoes were caught in March. *A. maculatus* was again scarce, however, *A. aconitus* and *A. philippinensis* were the most prevalent now. It is interesting to note that in October 7% of the mosquitoes captured were *Anopheles spp.*, whereas in March, 22% were. *Culex spp.* were the most prevalent mosquitoes caught in both trips; however, *Culex vishnui* was the main mosquito caught in October, but *Culex tritaeniorhynchus* predominated in March.

Light traps in the canopy captured the most mosquitoes in October; whereas, the human collectors on the jungle fringe caught the greatest number in March. 28% of the mosquitoes caught at the indoor-outdoor collection site were captured indoors during October, and 36% in March. More anophelines were found indoors in March despite several cycles of residual spraying. 1445 anophelines were dissected and 495 (or 34%) were found to be parous. There were 16 out of 77 *Mansonia crassipes* found to be parous. No positives were seen.

Anopheles barbirostris, *A. hyoanus* group, *A. karwari*, *A. kochi*, *A. philippinensis*, and *A. vagus* were found during the larval surveys. Ten species of culicines were found breeding at the same sites. No *A. balabacensis* were found.

Malaria in Jungle-dwelling People

Background: The northcentral part of West Malaysia is primarily mountainous and sparsely inhabited (4 people per square mile).

The jungle-dwelling people in these areas are called *Orang Asli* or Aborigines. Medical care for these *Orang Asli* is the responsibility of the Aborigine Hospital at Gombak, Selangor. In collaboration with the hospital's senior medical officer, we are studying malaria in these jungle-dwelling people, comparing deep jungle areas to fringe jungle. Blood surveys to determine malaria prevalence among the inhabitants in these areas are conducted periodically. Deep jungle villages in the Fort Kemar area and the Pos Shean have been monitored for changes in malaria prevalence due to weekly drug prophylaxis (chloroquine + pyrimethamine) and trimonthly spraying with residual insecticide (DDT). Fringe villages and schools near 7th mile Cameron Highlands Road and Satak have also been monitored. See map for the relative locations of these villages.

Pos Shean and Fort Kemar are surrounded by deep jungle and are completely isolated by mountains ranging up to 7000 feet in elevation. Travel into these areas is feasible only by helicopter. Difficulties in getting helicopter support for the Kemar area have curtailed surveys at the Fort Kemar village. Satak and Woh are accessible by 4-wheel drive vehicles and are located near the roadside. The people living in the deep jungle areas hunt and fish daily for food; whereas, the fringe dwellers buy their food from stores.

A study was conducted at Pos Shean in December to determine if the positive falciparum cases detected in November had chloroquine-resistant malaria. Resistant malaria was suspected as there was an increase in the prevalence of malaria among these people even though drug prophylaxis was being given out. A survey was conducted at Pos Legap, Perak (See Map) to determine if logging and road building in this formerly deep jungle area had increased the amount of malaria in these *Orang Asli.*

Mosquito surveys were conducted in Pos Shean, Pos Legap, and Woh Pos (and 14th mile Cameron Highlands Road) to determine the primary malaria vectors in the study areas and to compare the mosquito fauna in deep jungle with that on the fringe. At Pos Shean bare leg collections and indoor-outdoor collections were conducted. At Pos Legap light traps (with dry ice) placed at ground level and in the canopy were also used. In addition to these methods human bait traps were used at Woh and 14th mile Cameron Highlands Road. Larval surveys were made at all the study sites. Collections were made for 3 nights at Pos Shean and Pos Legap. Two trips were made to Woh and 14th mile Cameron Highlands Road--each for 4 nights.

    Results:

Human Studies: Five surveys were conducted in deep jungle villages--four at Pos Shean and one at Fort Kemar. Logistical problems in the Fort Kemar area have curtailed any further studies at this village. In June 1971, a survey was conducted at Pos Shean and 119 individuals were examined. 4 *Plasmodium falciparum* and 2 *P. vivax* cases were detected (5% positive). 192 people were surveyed in November 1971 with 13% being found positive--28 *P. falciparum,* 5 *P. vivax,* and 1 mixed infection. As this increase of malaria indicated either a breakdown in the weekly drug prophylaxis and the residual spraying program or resistance of the parasites, a study was made of these positives in December. Out of 32 people, 21 were positive on the first day of treatment. All individuals received 25 mg. of chloroquine per kg. of body weight over a 3-day period. Urines were tested with Mayer's Reagent before treatment and on the last day of treatment. All urines were negative on Day 0 and positive on Day 2. Blood films were again taken on Day 7. All were found to be negative, indicating that the falciparum malaria was either sensitive or resistant at an R-1 level. Therefore, it is felt that the high positivity ratio in November was due to a breakdown in drug distribution rather than to resistance of the falciparum parasites. In February 1972, 254 blood films were made with 8% being found positive (17 *P. falciparum,* 2 species undetermined, and 1 *P. vivax*). A survey was conducted in the Fort Kemar area in July 1971 with only 34 people giving blood during a three-day period. One individual had a *P. falciparum* infection (3% positive).

Four malaria surveys were made at the fringe village areas - Satak and 7th mile Cameron Highlands Road. In November 1971 at Satak, 132 people were examined and 19 (14%) were found to be positive. 10 were *P. vivax*, 4 were *P. falciparum*, 2 were *P. malariae*, and 3 were mixed infections. During February 1972 another 126 slides were taken with 2% being positive--1 *P. falciparum* and 1 *P. vivax*. A survey was conducted at a school located on 7th mile Cameron Highlands Road in July 1971. 74 schoolchildren were examined and 9 (12%) were found to have malaria parasites in their blood (7 were *P. falciparum* and 2 were *P. vivax*). At the same school in May 1972, 89 schoolchildren were examined. Only 3 children, or 3% positive, had malaria. All of these infections were *P. falciparum*.

In Table 4 all the survey results from Fort Kemar, Pos Shean, Satak, and 7th mile Cameron Highlands Road are listed. Also shown are the number of spraying cycles (trimonthly with DDT) and number of months prophylaxis has been given out (weekly chloroquine + pyrimethamine). It appears that these control measures are having an effect on the malaria prevalence among these jungle-dwelling people. The jungle fringe villages have lower rates now than in 1970. The amount of malaria at Fort Kemar apparently is going down but the numbers involved are too small to be sure. At Pos Shean the malaria prevalence went down initially but went up again with a breakdown in drug distribution. Supervision is the key to the success of this program. Getting small children to take any prophylaxis is very difficult. Most of the positive cases of malaria detected during this year were in children of less than 10 years of age. The effect of residual spraying is greatly limited by the type of house that these people live in (bamboo and atap) and by the frequency with which these people are away from home at night hunting or fishing. A third of their nights are spent in temporary shelters built on the ground or in the trees. The fringe dwellers spend much less time away from home and live in more permanent homes.

One survey was conducted at Pos Legap in April 1972. 65 people were examined and 3% were found to be positive (1 *P. falciparum* and 2 *P. vivax*). However, only 59 of these people were from this particular area, so the positivity ratio is actually 5%. This is a much lower percentage than 2 weeks before when 60 people were examined by some of the hospital staff from Gombak, and 25% were found to be positive. A special effort was made to give out chloroquine + pyrimethamine then; hence, the lower amount of malaria 2 weeks later.

Mosquito surveys: From 28 to 30 March 1972 a collection trip was made at Pos Shean. 959 adult mosquitoes were collected. No anophelines were caught as adults, however, *Anopheles maculatus* larvae were found breeding in rock pools alongside the river. Most of the culicines captured belonged to the genus *Armigeres*, of which 11

Table 4

Results of Blood Surveys for Malaria among the *Orang Asli* to Evaluate the Effect of Trimonthly Residual Spraying (DDT) & Weekly Prophylaxis (Chloroquine + Pyrimethamine)

Location	Date	Total No. Ex.	Total No. + (%)	P. falciparum	P. vivax	P. malariae	Mixed	S.U.*	No. of Spraying Cycles	No. of Months on Prophylaxis
Fort Kemar (Deep jungle village)	March 1970	97	9(9)	8	-	1	-	-	0	0
	July 1970	57	6(11)	3	1	1	-	-	1	3
	December 1970	36	0(0)	-	-	-	-	-	2	6
	July 1971	34	1(3)	1	-	-	-	-	4	12
Pos Shean (Deep Jungle village)	May 1970	115	25(22)	22	-	3	-	-	0	0
	October 1970	136	28(21)	20	5	1	-	-	1	3
	February 1971	171	4(2)	4	-	-	-	-	1	7
	June 1971	119	6(5)	4	2	-	-	-	2	7
	November 1971	192	34(18)	28	5	-	1	-	3	11
	December 1971**	32	21(-)	21	-	-	-	-	5	16
	February 1972	254	20(8)	17	1	-	-	2	5	17
Satak (Fringe jungle village)	September 1970	52	13(25)	8	1	4	-	-	1	2
	November 1971	132	19(14)	4	10	2	3	-	5	16
	February 1972	126	2(2)	1	-	1	-	-	6	19
7th Mile Cameron Highlands Road (Fringe jungle village)	September 1970+	55	20(36)	12	2	4	2	-	1	19
	November 1970++	74	41(55)	20	6	-	15	-	1	1
	November 1970+++	52	23(44)	17	2	-	4	-	1	3
	July 1971++	74	9(12)	7	2	-	-	-	4	12
	May 1972++	89	3(3)	3	1	-	-	-	7	22

* S.U. - species undetermined
** Positive cases from November 1971 were reexamined as part of a chloroquine-resistant malaria survey.
+ Noh Pos - near 7th Mile Cameron Highlands Road
++ School at 7th Mile Cameron Highlands Road
+++ School at 14th Mile Cameron Highlands Road

species were caught. 78 out of 447 adults caught at the three indoor-outdoor collection sites were found indoors. A summary of the collection results is shown in Table 5.

A mosquito survey was made at Woh Pos and 14th mile Cameron Highlands Road from 27-30 December 1971. A total of 1232 mosquitoes (Table 6) were caught, of which 106 were *Anopheles spp.* Half of the anophelines were *A. maculatus.* More mosquitoes were caught indoors at the indoor-outdoor collection sites than outdoors, including more *A. maculatus.* The houses were reported to have been sprayed. 3 *A. leucosphyrus* were also found indoors. *Culex vishnui* subgroup, *Armigeres (L.) flavus,* and *Armigeres (L.) annulitarsis* were the main culicine species captured. 33 out of 89 anophelines were parous, but no positive mosquitoes were found.

Another survey was made at Woh Pos and 14th mile Cameron Highlands Road from 4-7 April 1972. It was extremely dry in the area at the time, and only 838 mosquitoes were collected. 20 species of culicines were captured but only 1 anopheline species--*A. maculatus.* Both of the *A. maculatus* were negative. *Culex vishnui* subgroup, *C. nigropunctatus,* and *Mansonia dives* were the main species collected. More of the *Culex* were captured in the canopy light traps than in the ground ones. 23% of the mosquitoes caught at the two indoor-outdoor collection sites were captured indoors. See Table 7 for a summary of the results.

Twenty-two species of mosquitoes were collected from 25-27 April 1972 at Pos Legap. A total of 768 adults were caught, with all of the *Anopheles spp.* being caught during the bare leg collections. No anophelines were found in the light traps. Heavy wind and rain affected the operation of the light traps. Of 20 *A. maculatus* caught biting the collectors, 2 were found to be positive for oocysts. No sporozoites were seen, however, both mosquitoes had partially engorged on the collectors. The oocysts appeared to be mature. One *A. barbirostris* and 1 *A. montanus* were captured indoors. *Aedes spp.* and *Culex spp.* were caught in the most abundance. Many anopheline larvae were found in the blocked streams beside the logging road. This increased availability of breeding sites may affect the amount of malaria in the area. Table 8 summarizes the adult mosquito collection results.

Studies on Jungle Canopy Mosquitoes

Bukit Lanjan:

Background: Baseline studies comparing the mosquito fauna in the jungle canopy with the fauna at ground level were concluded during this reporting period. Extensive logging operations were started in the study area in the latter part of January, and it was felt that the

Table 5

Summary of Mosquito Collection Results at Pos Shean, Pahang,
28-30 March 1972

Species Collected	Method of Collection			Total
	Bare Leg Collection	B.L.C.		
		Indoors	Outdoors	
Aedes albopictus	60	6	16	82
Aedes niveus group	6	-	4	10
Aedes sp.	1	-	1	2
Armigeres (L.) annulitarsis	156	17	78	251
Armigeres (L.) balteatus	-	-	5	5
Armigeres (L.) dentatus	-	-	2	2
Armigeres (L.) digitatus	3	-	-	3
Armigeres (L.) dolichocephalus	24	4	20	48
Armigeres (L.) flavus	256	46	228	530
Armigeres (L.) inchoatus	1	-	1	2
Armigeres (L.) longipalpis	-	2	7	9
Armigeres (L.) magnus	2	1	6	9
Armigeres malayi	1	-	-	1
Armigeres (L.) pectinatus	2	2	1	5
Total	512	78	369	959

Table 6

Summary of Mosquito Collection Results at Woh Pos and
14th Mile Cameron Highlands Road, Perak,
27-30 December 1971

Species Collected	Method of Collection				Total
	Bare Leg Collection	B.L.C. Indoors	B.L.C. Outdoors	Human Bait Trap	
Anopheles aitkeni	1	-	-	-	1
Anopheles karwari	11	4	1	33	49
Anopheles leucosphyrus	-	3	-	-	3
Anopheles maculatus	15	13	7	18	53
Aedes (Aedes) sp.	2	-	-	-	2
Aedes albopictus	27	4	-	21	52
Aedes niveus group	1	1	2	1	5
Armigeres (L.) annulitarsis	52	47	33	16	148
Armigeres (L.) dolichocephalus	1	1	-	-	2
Armigeres (L.) flavus	62	59	28	47	196
Armigeres (L.) nagnus	-	1	-	-	1
Armigeres malayi	3	1	6	-	10
Culex gelidus	7	3	9	30	49
Culex (Lopho.) spp.	4	4	1	6	15
Culex nigropunctatus	5	2	3	7	17
Culex vishnui subgroup	169	47	74	225	515
Mansonia dives	50	2	18	43	113
Mansonia uniformis	1	-	-	-	1
Total	411	192	182	447	1232

Table 7

Summary of Mosquito Collection Results at Woh Pos and 14th Mile
Cameron Highlands Road, Perak, 4-7 April 1972

Species Collected	Method of Collection					Total
	Bare Leg Collection	B.L.C.		Light Trap($W.CO_2$)		
		Indoors	Outdoor	Ground	Canopy	
Anopheles maculatus	-	-	1	-	1	2
Aedes albopictus	22	2	20	-	2	46
Aedes niveus group	3	-	6	10	1	26
Armigeres (L.) annulitarsis	-	-	2	-	-	2
Armigeres (L.) digitatus	2	-	-	-	-	2
Armigeres (L.) dolichocephalus	-	-	1	-	-	1
Armigeres (L.) flavus	4	-	-	-	-	4
Armigeres (L.) longipalpis	-	-	1	-	-	1
Armigeres (L.) omissus	1	-	2	-	-	3
Armigeres (L.) pectinatus	-	-	1	-	-	1
Armigeres subalbatus	39	10	23	-	2	74
Armigeres (L.) traubi	-	-	1	-	-	1
Culex culiciomyia	-	-	-	-	1	1
Culex fatigans	1	-	-	-	-	1
Culex gelidus	10	4	5	1	-	20
Culex (Lopho.) spp.	-	-	5	20	17	42
Culex nigropunctatus	14	-	14	22	37	87
Culex vishnui subgroup	79	26	72	60	117	354
Mansonia crassipes	-	-	-	-	1	1
Mansonia dives	53	16	47	25	18	159
Mansonia uniformis	3	2	-	3	2	10
Total	237	60	201	141	199	838

Table 8

Summary of Mosquito Collection Results at Pos Legap, Perak,
25-27 April 1972

Species Collected	Method of Collection					Total
	Bare Leg Collection	B.L.C.		Light Trap($W.CO_2$)		
		Indoors	Outdoors	Ground	Canopy	
Anopheles barbirostris	5	1	-	-	-	6
Anopheles montanus	-	1	-	-	-	1
Anopheles maculatus	15	-	5			20
Anopheles philippinensis	6	-	-	-	-	6
Anopheles tessellatus	1	-	-	-	-	1
Aedes spp. (4)*	136	18	29	.	36	221
Armigeres spp. (4)*	5	4	1	-	-	10
Culex spp. (5)*	304	5	51	60	51	471
Mansonia spp. (3)*	7	-	-	-	2	9
Uranotaenia sp. (1)*	-	-	-	23	-	23
Total	479	29	96	85	89	768

* Number of different species

basic studies begun two years ago should be concluded. The logging operations were likely to change the breeding habitats for larval mosquitoes, and thus the adult fauna found at both ground level and within the canopy. Therefore, collections made during the last two years could not be compared on a equal basis with the collections made subsequent to logging and selected cutting. However, studies with different goals (e.g. resting sites of mosquitoes in the canopy, host blood meal identification, and arbovirus isolation) can validly be investigated in the future at this site.

The study area is located about 12 miles west of Kuala Lumpur near an aborigine settlement. The site itself is a small area of primary forest on the side of a hill which is interspersed with small streams and seepages. Trees 80 to 170 feet tall are found throughout the area. The logging operations took out many of these taller trees. Much low-growing scrub is found at ground level. The area may now be classified as disturbed primary forest. A system of aluminium ladders has been set up within the canopy to form a walkway. The walkway is constructed horizontally from the hillside using trunks as vertical supports. It is possible for a person to walk along this transect for more than 1200 feet through the canopy at heights above the ground ranging from 20 to 130 feet. Twelve platforms have been built at various points along the walkway. Meteorological equipment for measuring relative humidity, temperature, and rainfall are placed both in the canopy and at ground level.

Human biting collections (bare leg collections), CDC light traps with solid CO_2, and oviposition trap collections were used from July 1971 through February 1972. Weekly collections from the oviposition traps are continuing. Black glasses were checked daily during January 1972. Human biting collections were made by four men--two on platforms and two on the ground--from 1800 to 2230 hours. Nine light traps were placed in the canopy and nine at parallel points on the ground. Traps were turned on at 1800 hours and turned off at 0730 hours. Collection bags are brought back to the laboratory for processing of mosquitoes. All mosquitoes are identified and many individual rearings were made from the oviposition trap collections to facilitate taxonomic determinations. *Anopheles spp.* are dissected for the detection of malaria parasites. *Mansonia crassipes* are also dissected to determine the presence of filarial worms. (See Annual Report, 1970, for collection site locations).

Ampang Reservoir: Because of the possibility of the Bukit Lanjan transect system having to be taken down due to deforestation in the area, another possible canopy study area was examined. The area is part of one of Kuala Lumpur's water catchment areas located near the city's eastern boundary. As this area is protected against logging operations, a transect system could be put up without fear of it being disturbed by housing development or logging firms. The area is a

primary forest reserve and is only about 15 minutes from the USAMRU laboratory. Daily collections of mosquitoes could easily be carried out on a routine basis without much time being spent driving to and fro. Many monkeys and gibbons have been observed in the area (Bukit Lanjan had only a few). As a new suburban development is located on the fringe of the forest, it is possible that a monkey-mosquito-man interrelationship could occur. This area offers the opportunity to study the possible flow of disease from canopy-dwelling animals to ground-dwelling animals (including man) via a dipteran vector.

Bare leg collections and CDC light trap collections were made in this study area in May 1972 to determine the potential vectors. The light traps were baited with solid CO_2 and were placed both at ground level and 20-30 feet above the ground. The traps were turned on at 1730 hours and turned off at 0730 hours. Bare leg collections were made from 1800 to 2230 hours.

Results:

Bukit Lanjan: A total of 25 species of mosquitoes were collected at both ground and canopy level. Over 3400 adults were caught in light traps and almost 3100 in human biting collections. (See Table 9 and 10.) Only 90 anophelines were captured and only 12% of these were found at ground level. Overall, 63% of the adults were collected at the ground level sites. In both light trap and human biting collections, *Culex vishnui* subgroup was the main mosquito captured. Of the *Anopheles spp.*, *A. riparis* and *A. leucosphyrus* were caught most frequently. Both of these species were caught almost entirely at canopy level. One specimen of *A. riparis* did try to feed on man at ground level. *Aedes albopictus* was collected mostly at ground level, whereas, *Mansonia crassipes* was caught more frequently at canopy level. *Mansonia dives* was found more often at canopy level in the higher canopy than in the lower canopy.

Ten species of mosquito larvae were found breeding in bamboo cups or black glasses at Bukit Lanjan. *Aedes albopictus* and *Armigeres spp.* were found both at ground level and within the canopy oviposition traps. *Aedes pseudoniveus* was breeding only at canopy level - whereas, *Aedes jugraensis*, *Aedes harveyi*, *Aedes chrysolineatus* group, *Culex brevipalpis*, *Culex (Lopho.) spp.*, *Toxorhynchites spp.*, and *Tripteroides affinis* were found breeding only at ground level.

Dissection results of adult mosquitoes at Bukit Lanjan were as follows:

73 anophelines dissected	637 culicines dissected
29 parous (40%)	181 parous (28%)
0 positive	7 positive (filarial worms)

Table 9

Summary of Light Trap Collection Results,
Fringe Forest & Deep Forest, Bukit Lanjan,
from July 1971 to February 1972

Species Collected	Fringe Forest		Deep Forest		Total
	Ground Level	Canopy Level	Ground Level	Canopy Level	
Anopheles aitkeni	1	-	-	-	1
Anopheles leucosphyrus	-	-	-	19	19
Anopheles riparis	-	27	1	15	43
Anopheles watsonii	-	1	-	-	1
Aedes albopictus	4	-	2	-	6
Aedes chrysolineatus	1	-	-	-	1
Aedes niveus group	2	11	-	3	16
Aedes prominens	-	-	3	-	3
Aedomyia catastiota	-	19	-	1f	35
Armigeres (L.) flavus	1	-	-	-	1
Armigeres subalbatus	1	-	1	-	2
Culex gelidus	-	1	2	1	4
Culex (Lopho.) spp.	2	7	3	9	21
Culex nigropunctatus	12	1	8	1	22
Culex vishnui subgroup	1491	283	438	6	2274
Mansonia crassipes	209	374	59	86	728
Mansonia dives	40	58	60	91	249
Mansonia nigrosignata	-	1	1	-	2
Mansonia ochracea	9	-	1	-	10
Orthopodomyia sp.	-	1	-	-	1
Total	1773	784	579	303	3439

Table 10

Summary of Human Biting Collection (B.L.C.) Results,
Fringe Forest and Deep Forest, Bukit Lanjan,
from July 1971 to February 1972

Species Collected	Fringe Forest		Deep Forest		Total
	Ground	Platform	Ground	Platform	
Anopheles aitkeni	1	-	1	-	2
Anopheles interruptus	1	-	-	-	1
Anopheles letifer	1	1	4	-	6
Anopheles leucosphyrus	-	-	-	2	2
Anopheles riparis	-	-	1	3	4
Anopheles watsonii	-	5	-	-	5
Aedes albopictus	78	4	57	3	142
Aedes (F.) albotaeniatus	1	-	-	-	1
Aedes chrysolineatus	-	-	1	-	1
Aedes khazani	1	-	-	-	1
Aedes niveus group	4	4	-	1	9
Aedes promixens	10	1	⟨	1	17
Armigeres (L.) flavus	6	-	-	-	6
Armigeres malayi	15	-	2	-	17
Armigeres subalbatus	104	2	82	7	195
Culex gelidus	46	1	4	5	56
Culex (Lopho.) spp.	3	1	7	-	11
Culex vishnui subgroup	1190	264	628	187	2297
Mansonia crassipes	-	12	1	17	30
Mansonia dives.	106	37	66	70	279
Total	1567	362	837	236	3082

Ampang Reservoir: In the small survey done at Ampang only 813 adults were collected, 53 by bare leg collections and 760 by light traps. Most of mosquitoes captured in light traps were caught in the canopy (81%). Only 6 anophelines were collected, but of these *Anopheles b. introlatus, A. crawfordi,* and *A. leucosphyrus* are considered to be vectors of simian malarias. Five hundred *Mansonia crassipes* were caught and dissected; of these, 143 were parous. Oocysts were found in one specimen and filarial worms in six. A summary of collection results are found in Table 11.

Studies of Tragulid Malaria

As monkeys are expensive and rodent malarias are difficult to transmit by sporozoites, we have started studying tragulid malaria as a possible laboratory model for the study of the exoerythrocytic system. *Plasmodium traguli* can be found in the liver of naturally infected animals, mosquitoes can be infected easily, and most of the tragulid hosts are infected at the time of capture. From these data it appears that this should make an excellent laboratory model. Although previous workers were hindered by unsuccessful colonization of the host, we have been successful in maintaining and breeding these animals in captivity.

During this reporting period both electron and light microscopy studies were made of the erythrocytic forms of *Plasmodiu.. traguli. Anopheles barbirostris, A. letifer,* and *A. maculatus* were fed on infected hosts. Studies were then made on the exogenous phase of *P. traguli.* Infected glands were injected IP into laboratory hosts that had been born in captivity. Liver sections from wild-caught animals were stained and examined for exoerythrocytic forms. Liver sections were fixed in either Carnoy's or Formalin and stained with Colophonium Giemsa or H&E. Sporozoites were fixed in either Carnoy's methanol, Bouins, or iodine vapor and stained witn Methyl Green Pyronin, P.A.S., Heidenheins hematoxylin, Ag protein, or Giemsa. Oocysts were fixed in Carnoy's Fluid or Formalin and stained with Methyl Green Pyronin or Mercurochrome. Sporozoites and oocysts were also fixed ror election microscope studies. Daily blood films from the infected hosts were fixed in methanol and stained with Giemsa.

Results: Studies of the erythrocytic stages of *P. traguli* by both electron and light microscopy showed that certain animals had two species of *Plasmodium (P. traguli* and *P. n.sp.).* As evidenced by light microscopy, the erythrocytes of *P. traguli* are very minute, about 1.70 to 3.0 µ in diameter--roughly one-eighth the size of normal human erythrocytes. The erythrocytes exhibit very little or no enlargement when parasitized by the asexual and sexual stages of *P. traguli.* The earliest asexual forms seen are rings with a nucleus and vacuole occupying 1/3 of the host blood cell. As the trophozoites grow larger, the vacuole disappears and a tiny grain of yellow pigment

Table 11

Summary of Mosquito Collection Results at Ampang Reservoir, Selangor,
May 1972

Species Collected	Bare Leg Collection	Light Trap(W.CO_2) Ground	Canopy	Total
Anopheles balabacensis introlatus	-	-	1	1
Anopheles crawfordi	3	-	-	3
Anopheles indiensis	1	-	-	1
Anopheles leucosphyrus	1	-	-	1
Aedes albopictus	-	-	1	1
Armigeres subalbatus	-	1	-	1
Culex bitaeniorhynchus	1	1	-	2
Culex gelidus	2	-	-	2
Culex (Lopho.) sp.	-	3	12	15
Culex nigropunctatus	-	5	12	17
Culex vishnui subgroup	37	77	125	239
Mansonia crassipes	1	51	448	500
Mansonia dives	7	1	-	8
Mansonia ochracea	-	5	13	18
Orthopodomyia sp.	-	-	1	1
Uranotaenia campestris	-	-	1	1
Uranotaenia testacea	-	-	1	1
Uranotaenia trilineata	-	1	-	1
Total	53	145	615	813

appears in the cytoplasm. The schizonts can be distinguished by the presence of fairly prominent nuclei, scanty cytoplasm, and a single pigment grain. 4-8 merozoites are seen in the mature schizont. Gametocytes are about 3.5 μ in diameter and almost fill the host erythrocytes. A prominent nucleus and a larger pigment grain in the cytoplasm are easily seen in both the micro- and macro-gametocytes. The sex may be distinguished by the color of its cytoplasm (pale blue for the females and pinkish for the males).

Other than *P. traguli* we have found a new species of *Plasmodium* that is much larger than the already described species. The new parasite measures 6.5 μ x 8 μ to 8 μ x 10 μ. Its shape is ovoid, spherical, angular, or irregular in outline. The organism appears to assume two or more forms. In one form it contains multiple nuclei with vacuolated pale cytoplasm and suggestion of one or two tiny grains of faint yellowish pigment that glow when examined under polarized light. Another form contains a single large mass of nucleus, sometimes with dense chromatin granules distributed throughout the vacuolated cytoplasm, with two or more minute grains of faint yellowish pigment. Occasionally there is a suggestion of a pale pinkish cellular background or outline. Out of 86 tragulid hosts examined, 64 (74%) had *P. traguli* and 12 (14%) had the new large parasite.

During the last 9 months as animals showed gametocytes in their peripheral blood smears, mosquitoes were allowed to feed. For some reason the greatest number of gametocytes were seen in September, October, and November. Gametocyte carriers in subsequent months were extremely rare although the hosts' blood continued to show asexual forms in good abundance. Dissection results are summarized in Table 12. The earliest that sporozoites were seen was on Day 8. Sporozoites were most frequently seen on Day 11. Although one group of *A. barbirostris* developed oocysts, none exhibited sporozoites. Up to 67% of the *A. letifer* were positive for sporozoites, and up to 79% of the *A. maculatus*.

We fed mosquitoes *(A. letifer)* on one mouse deer that had both *P. traguli* and the large parasites in its blood. Gametocytes of both species were present at the time of feeding. 18 days later 4 infected glands were inoculated into a mouse deer which had been born and raised in captivity. On the 11th and 12th days after inoculation the large parasite was seen in the blood films. By the 13th day *P. traguli* was also seen. This suggested that the anopheline mosquito can transmit these new parasites, but further transmission studies need to be carried out. In another transmission experiment a baby mouse deer that had been born in captivity became positive 82 days after receiving an IP inoculation of 5 infected glands of *A. maculatus*. In this instance only *P. traguli* was seen. Another mouse deer which received 2 glands of *A. maculatus* became positive 11 days later.

Table 12

Summary of Mosquito Dissection Results,
Host: *Tragulus javanicus*
Parasite: *Plasmodium traguli* & *Plasmodium n.sp.*

Species of Mosquito	Total No. Dissected	Per Cent Positive		Host No.
		Oocysts	Sporozoites (Day First Seen)	
Anopheles barbirostris	3	0	0	#97
	5	0	0	#100
	17	6	0	#113
	4	0	0	#113
Anopheles letifer	12	42	29 (D10)	#97
	14	64	67 (D9)	#97
	3	0	0	#84
	33	0	0	#47
	46	2	0	#144
	53	0	0	#132
	5	40	0	#147
Anopheles maculatus	22	55	18 (D11)	#97
	25	60	8 (D14)	#100
	53	58	77 (D11)	#101
	49	53	79 (D11)	#97
	40	38	47 (D8)	#97
	114	15	13 (D12)	#97
	69	25	13 (D14)	#113
	55	11	0	#113
	3	33	0	#84
	67	3	0	#144
	'J	3	0	#132
	85	13	8 (D11)	#147
	65	15	7 (D13)	#147

Further studies are being made on the sporogonic stages and exoerythrocytic stages using the electron microscope. A formal publication of the erythrocytic stages of *P. traguli* using the electron microscope is being prepared. A description of the new parasite will be made during the next year.

Studies on Primate Malarias

In support of the experiments on the exoerythrocytic cycle in liver tissue culture, mosquitoes were fed on monkeys infected with *Plasmodium cynomolgi*. *Anopheles maculatus* were fed mainly on pig-tailed macaques; however, the original strain of *P. cynomolgi* came from a Rhesus monkey. Mosquitoes were usually fed on animals whose blood contained at least one male gametocyte and one female gametocyte per 100 WBC. Results of the feedings and dissections are shown in Table 13. The most successful feeding was with a group of 44 *A. maculatus* that fed on p.t.-55. This monkey had a parasitemia of 32,580 asexual parasites per cmm. of blood and 4♀♀/2♂♂ per 100 WBC. 98% of the mosquitoes developed oocysts, and 93% developed sporozoites (beginning on Day 10).

Results of spprozoite inoculations into tissue culture are discussed in another section of this report.

A gibbon *(Hylobates lar)* was caught in the jungle in Negri Sembilan by an *Orang Asli*. When the animal was brought to our lab, he was anemic and was suffering from a bacterial infection. A blood film was taken to see if the gibbon also was infected with malaria. The blood film was positive for *Plasmodium youngi*. Realizing that only the erythrocytic stages had been studied, we began to study this parasite. Other gibbons were infected by either blood inoculation or sporozoite inoculation. Daily blood films were taken from all of the infected gibbons and liver sections were taken from gibbons that had received sporozoites. Biopsies were made on Days 5, 7, 8, and 10 following inoculation. Sections were stained with Price's Giemsa, Colophonium Giemsa, and H&E. Blood was fixed for electron microscopy. Periodicity studies were made over a week's period of time at 4 hourly intervals. Mosquitoes *(Anopheles letifer, A. indiensis,* and *A. maculatus)* were fed on hosts that had sexual forms of *P. youngi* in their blood. Table 14 summarizes the results of the mosquito dissections.

Mosquito Colonies

In order to support various studies being conducted by our lab, mosquitoes are being reared in an indoor and in an outdoor insectary. Indoors the temperature stays at 78°F ± 2° and the relative humidity runs at about 85% ± 3%. Adults are caged in small screened cages, and anopheles are mated artifically. Eggs are laid either in paper cups

Table 13

Summary of Mosquito Dissection Results
Host: *Macaca spp.*
Parasite: *Plasmodium cynomolgi*
Vector: *Anopheles maculatus*

Total No. Dissected	Asexual Parasites per cumm of Blood	No. of Gametocytes per 100 WBC(♀♀/♂♂)	Per Cent Positive		Host* No.
			Oocysts	Sporozoites (Day First Seen)	
183	20,000	1/1	92	77 (D11)	R-A117
117	89,400	1/1	83	49 (D10)	p.t.-M1
150	103,200	38/36	86	62 (D9)	p.t.-54
60	9,900	3/5	22	0	p.t.-M1
60	11,260	–	0	2 (D10)	p.t.-54
45	4,110 (P. *inui*)	3/1	51	0	R-1
29	44,700	1/2	90	79 (D10)	p.t.-55
44	32,580	4/2	98	93 (D10)	p.t.-55
173	22,200	2/2	94	91 (D10)	p.t.-55
82	4,080	–	18	12 (D12)	p.t.-53
90	7,080 (P. *inui*)	1/1 (per 150 WBC)	44	0	R-1
81	<500 (P. *inui*)	–	1	0	l.t.-C1
115	4,890	2/3	87	67 (D10)	p.t.-55
110	5,490	4/2	88	67 (D10)	p.t.-55
40	11,790	1/1	0	0	p.t.-M1
35	36,900	3/1	0	0	p.t.-M1
76	13,950	1/3	94	31 (D11)	p.t.-55
58	102,450	5/2	88	24 (D12)	p.t.-68
50	221,250	15/9	0	0	p.t.-68
55	144,000	10/6	4	0	p.t.-68
80	42,480	3/1	60	24 (D11)	p.t.-68
109	44,880	2/1	90	37 (D10)	p.t.-68
70	45,030	2/1	74	27 (D11)	p.t.-68
110	48,720	♀♀ 8/50 WBC ♂♂ 4/100 WBC	92	33 (D10)	p.t.-68
70	12,510	1/2	0	0	p.t.-69

* Host: R-Rhesus; p.t.-Pig-tail macaque; l.t.-long-tailed macaque

Table 14

Summary of Mosquito Dissection Results,
Host: *Hylobates lar*   Parasite: *Plasmodium youngi*

Total No. Dissected	Asexual Parasites per cmm of Blood	No. of Gametocytes per 100 WBC (♀♀/♂♂)	Per Cent Positive Oocysts	Sporozoites (Day First Seen)	Host No.
Anopheles letifer					
51	-	-	0	0	G-12
79	34,860	1/1	18	5 (D12)	G-10
Anopheles indiensis					
22	34,860	1/1	0	0	G-10
Anopheles maculatus					
77	-	-	0	0	G-12
61	3,150	3/1	0	0	G-10
51	23,880	0/5	45	30 (D12)	G-10
80	36,390	5/2	79	60 (D10)	G-12
80	53,620	♀♀ 1/10 ♂♂ 2/10 WBC WBC	70	22 (D12)	G-12
140	57,960	♀♀ 2/20 ♂♂ 3/20 WBC WBC	54	13 (D16)	G-12
73	140,460	♀♀ 2/10 ♂♂ 1/10 WBC WBC	14	0	G-12
	15,960	0/1			G-12
	28,830	9/4			G-7
	37,830	5/2			G-7
	2,790	0/2			G-12
	39,300	7/4			G-7
	2,820	1/1			G-12
	30,930	1/2			G-7

containing wet filter paper or in bamboo cups. Eggs are placed in
porcelain bowls containing rain water. First instar larvae are placed
in white enamel pans containing a small amount of sterile soil and
rain water. Larvae are fed on a mixture of rat chow (4 parts), baby
cereal (5 parts), yeast (1 part), and ground up dried-beef liver
(1 part). Pupae are placed daily within cages in porcelain bowls
containing rain water. Adults are allowed to feed on cloth wicks
soaked in a glucose: vitamin complex solution (2 g. glucose to 10 cc
dist H_2O to 0.2 cc vitamin complex). Adult mosquitoes obtain their
blood meals from guinea pigs, monkeys, or chickens.

The following colonies of mosquitoes are now being maintained:
Anopheles letifer (F-13), *Anopheles maculatus* (F-23), *Anopheles
indiensis* (F-12), *Anopheles peditaeniatus* (F-3), *Aedes albopictus*
(F-24), and *Aedes aegypti* (F-27). Other species are also being
individually reared for taxonomic studies.

Reference Collection

Several additional species of mosquitoes have been added to the
USAMRU reference collection. Emphasis this year has been on
obtaining associated larval and pupal skins as well as the pinned
adult. Larval and pupal skins are mounted on the same slide and are
given the same reference number as the associated pinned adult. The
skins are transfered from 70% alcohol into 75% alcohol for 20 minutes,
then into 95% alcohol for 20 minutes. From the alcohol they are
transfered into clove oil for 30 minutes and then into thin Euparal on
slides. The slides are then placed into an oven (55°C) for 30 minutes
without coverslips. Thick Euparol is then added and coverslips are
put on. The slides are then dried in the oven for 5 days. This
mounting method allows considerable manipulation of the specimens and
there is practically no loss of hairs.

Investigations of Malaria *in vitro*

Investigations on the Exoerythrocytic Stages of Malaria *in vitro*

In association with the normal liver tissue culture work, attempts
were made to reproduce *in vitro* the primary exoerythrocytic stage of
Plasmodium cynomolgi; as a model of this stage for studies of growth
requirements and drug testing. No successful *in vitro* reproduction of
this malarial stage in mammals has previously been published.

Salivary glands containing sporozoites of *Plasmodium cynomolgi*
were dissected from experimentally infected *Anopheles maculatus*, and
added to the liver explants after various periods of culture. Five
infected glands were added to each tube, which contained 4 pieces of
liver. At 8, 10, and 12 or 14 days after addition of the sporozoites,
liver tissue was inoculated intraperitoneally into a splenectomized

pig-tailed macaque *(Macaca nemestrina)*. Simultaneously some of the
liver tissues were prepared for histological examination and stained
with modified Price-Giemsa. Medium from the cultures after as little
as two days post addition of salivary glands was inoculated into a
control splenectomized monkey. The first liver recipient monkey had
been in the USAMRU colony for about 9 years and was negative for
malaria on numerous blood examinations. The others had been in the
colony for at least several years each. All monkeys were kept in
double screened quarters to avoid accidental mosquito borne
infection.

Histological preparations of the cultured liver were unsatisfactory,
possibly due to the friability of the infarcted cores of the liver.

In the first of the four completed experiments the "medium
recipient" monkey remained negative for patent malarial infection.
However, the tissue recipient became positive. Altogether it
received approximately 45 pieces of tissue in 15 intraperitoneal
inoculations. The first parasites appeared 32 days after splenectomy,
20 days after the first tissue inoculation, but only 6 days after the
last inoculation. This monkey had the appropriate *Plasmodium cynomolgi*
with a pattern of parasitemia consistent with a primary infection.
This monkey had been in the laboratory for more than four years prior
to this work.

In the second experiment, the tissue recipient was found to have
only three parasites, which appeared 9 days after the last inoculation
and 18 days after first tissue inoculation. This was greater than 2
months post splenectomy. While the three parasites were clearly
asexual malarial parasites, the species could not be identified with
certainty. The medium recipient monkey remained negative.

The subsequent two experiments, which had slight modification of
the liver culture technique, did not result in detectable parasitemia.

Further attempts are under way to reproduce the first findings.
Further modifications of the histological techniques are being
attempted.

Investigations on Drug Resistance of *Plasmodium falciparum in vitro*

Attempts are being made to modify the *in vitro* method of Diggs *et
al* *(J. Parasit.: 57*, 187-188, 1971) for field studies in Malaysia.
This work is in the preliminary stages. It is anticipated that this
method, if feasible, and the Rieckmann method already in use will be
employed in the field to determine what correlation might exist
between these *in vitro* systems and the parasitological and clinical
effect of the drug in the patient.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION	2 DATE OF SUMMARY 30 06 72	REPORT CONTROL SYMBOL DD-DR&E(AR)636

3 DATE PREV SUM'RY 30 06 71	4 KIND OF SUMMARY	5 SUMMARY SCTY U	6 WORK SECURITY	7 REGRADING N/A	8a DISB'N INSTR'N UL	8b SPECIFIC DATA CONTRACTOR ACCESS ☐ YES ☐ NO	9 LEVEL OF SUM A WORK UNIT

10 NO /CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a PRIMARY		3A062110A831		
b CONTRIBUTING				
c CONTRIBUTING				

11 TITLE (Precede with Security Classification Code)

Investigations of Scrub Typhus

12 SCIENTIFIC AND TECHNOLOGICAL AREAS

13 START DATE 10 71	14 ESTIMATED COMPLETION DATE 9 72	15 FUNDING AGENCY		16 PERFORMANCE METHOD	

17 CONTRACT/GRANT DADA17-72-G-9350			18 RESOURCES ESTIMATE	a PROFESSIONAL MAN YRS	b FUNDS (In thousands)
a DATE/EFFECTIVE 10 71	EXPIRATION 10 72		FISCAL 72	1.0	45.8
b NUMBER			YEAR		
c TYPE Y Grant	d AMOUNT 218		73	1.0	30.7
e KIND OF AWARD	f CUM. AMT.				

19 RESPONSIBLE DOD ORGANIZATION	20 PERFORMING ORGANIZATION
NAME US Army Medical Research Unit Institute for Medical Research Kuala Lumpur, Malaysia	NAME Institute for Medical Research Kuala Lumpur, Malaysia
RESPONSIBLE INDIVIDUAL NAME Dr. R. Bhagwan Singh, Acting Director TELEPHONE Institute for Medical Research	PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME Walker, J.S., MAJ, VC TELEPHONE: SOCIAL SECURITY ACCOUNT NUMBER
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22 KEYWORDS (Precede EACH with Security Classification Code)

Rickettsia, scrub typhus, leptotrombidium, R. tsutsugamushi

23.(U) Technical Objectives: To study the distribution of scrub typhus rickettsia in various tissues from each life stage of *Leptotrombidium (Leptotrombidium) fletcheri* (=akamushi) from the positive colony; to continue investigations of vector bionomics; to initiate a long term ecological study of scrub typhus vectors in a variety of adjacent habitats; to evaluate the silvered leaf-monkey as a primate model for human scrub typhus and determine the response to various strains and doses of *R. tsutsugamushi*; to determine if the gibbon is susceptible to scrub typhus; to continue the studies involving the antigenic stab'lity of *R. tsutsugamushi* in the three hosts (primates, as a model for man, the vector mites, and wild rodents); to determine if the vector mites can be infected from rodents; to determine the optimum conditions of freezing and storage of rickettsial organisms at high titer; to initiate studies on the effect of seasons on the isolation and serological rates of scrub typhus and infestation with vector chiggers in small mammals in various habitats and the effect of the immune or resistant status of the mammalian host on *R. tsutsugamushi* isolated from vector mites.

24.(U) Approach: Infected eggs and all stages of offspring from the positive *L. (L.) fletcheri* colony will be dissected and tissues will be examined for rickettsia by the direct fluorescent antibody technique. Optimum tissues for screening of known and potential vectors will be determined.

Egg production, rate of hatch and sex ratio of offspring of the positive and negative colonies of *L. (L.) fletcheri* will be compared.

DD FORM 1498

DD Form 1498, Research and Technology Work Unit Summary,
Item 24 Continued:

Chigger numbers will be monitored in a variety of adjacent habitats
using black plating and rodent trapping. Comparisons of relative
numbers with weather and host conditions will be made.

Attempts to infect chiggers by allowing them to feed on infected
rodents will be continued.

Silvered leaf-monkeys will be challenged with various strains and
combinations of strains at varying doses and closely followed
including a study of their gross and histopathology. The same
approach will be used for a few gibbons. Frozen material from the
positive *L. (L.) fletcheri* colony will be analyzed to determine what
strain(s) the colony has been and 's infected with if it or they
have remained antigenically stab. Organisms reisolated from
primates and rodents infected with known strains will also be
analyzed for antigenic stability. High titer suspensions of
organisms will be produced, frozen, stored and thawed under varying
conditions to determine the optimum. Studies will be initiated to
determine the effect of season and habitat on scrub typhus rates in
small mammals in a defined area where various habitats occur and
daily sampling done. To test the effect of the immune or resistant
status of the mammalian host on the virulence and antigenic stability
of *R. tsutsugamushi*, a single positive female from the positive mite
colony will be selected and three different lots of her offspring
will be fed on nonimmune white mice, immune white mice and
innately resistant *Callosciurus notatus* for three generations.

25.(U) <u>Progress</u>: Microdissection and the direct fluorescent antibody
technique were used to demonstrate scrub typhus rickettsia in 11
stages of infectious *Leptotrombidium (L.) fletcheri (akamushi)* from
the positive colony. The gut tissues and hemolymph were infectious
in all post egg stages. Unengorged larvae had the highest
percentage of tissues positive for rickettsia. Of eggs taken from
known infectious females, 91.7% were positive by FA examination.
Examination of egg contents with the FA technique appears to be a
feasible means for screening field collected vectors for
colonization, since the adult is kept alive.

The infectious *L. (L.) fletcheri* colony is into the 11th laboratory
generation. Observations of sex ratios in the infectious and non-
infectious colonies of this species suggest that a type of
parthenogenesis, possibly thelyotokus gynogenesis, is responsible
for the lack of males in the positive colony.

L. (L.) deliense numbers are being sampled in a variety of adjacent
habitats by black plate and rodent collections. Comparisons are
incomplete at present, but monthly fluctuations in numbers do not
consistently correspond to average monthly rainfall alone.

DD Form 1498, Research and Technology Work Unit Summary,
Item 25 Continued:

During some months, marked differences occurred in the average numbers
of chiggers on rodents and those on black plates. The number of
chiggers on male *Rattus argentiventer* and *R. tiomanicus jalorensis*
were notably higher than those on females of the same species. *R.
argentiventer* was the most important and *R. exulans* the least
important host species in the study area in terms of numbers of
chiggers per infested rat.

It was repeatedly demonstrated that larvae of the vector mites can
obtain rickettsial organisms from infected rodents (mice and rats).
However, to date, no transovarial transmission has occurred. The
efficiency with which mites take up rickettsia from infected rodents
appeared to be species dependent.

Silvered leaf-monkeys seem to be an excellent subhuman primate model
for human scrub typhus and all responses measured were strain and dose
dependent. Three strains failed to produce eschars at any dose (up to
10^6) while others produced eschars with doses as low as $10^{1.5}$.
Significant titers were obtained to the minor antigenic components of
the strains in addition to the major components. Complete protection,
as determined by clinical illness, was demonstrated in silvered leaf-
monkeys challenged at six months with homologous, homologous-
heterologous or heterologous combinations of strains. Immunity
affected both the formation and duration of eschar formation.

The organism was shown to be antigenically stable in silvered leaf-
monkeys, vector mites and a wild rodent. The minor Karp component of
Kato varies in its degree of expression both in vector mites and
silvered leaf-monkeys.

Storage studies of high titer material showed that within the limits
tested freezing and thawing rates had little or no effect on the
titers obtained and that through six months storage that there was no
significant differences between the titers of the material stored at
$-65^\circ C$ and $-175^\circ C$. Materials stored at both temperature lost 1 $\log_{10}$
of titer in six months.

An area was selected for a long term study of the effect of season and
habitat on mammalian isolation and serology ratios. Indicator species
for each habitat were selected and the study initiated for the
following habitats: a village area, edge habitat, lalang grass and
relict primary/secondary forest.

INVESTIGATIONS OF SCRUB TYPHUS

The investigations of scrub typhus vectors represent a combined effort by the Departments of Entomology and Rickettsial Diseases.

In previous Annual Reports, the name *Leptotrombidium (Leptotrombidium) akamushi* (Womersley 1952; Womersley and Audy 1957) was used in place of *L. (L.) fletcheri* (Womersley and Heaslip 1943). Vercammen Grandjean (1969) redesignated the Malaysian species as *L. (L.) fletcheri*, and Drs. Audy and Traub and Mr. M. Nadchatram have advised this unit of their acceptance of *L. (L.) fletcheri* as the proper name for the positive colony species. Another chigger, *Gahrliepia (Gahrliepia) fletcheri* Gater 1932 is also mentioned in the present report. It is unfortunate that 2 different species in the Trombiculinae have the same specific name.

Studies of Scrub Typhus Vectors

1. **Microdissection of Infectious Mites:** To determine the distribution of rickettsia in various tissues of each life stage of *Leptotrombidium (L.) fletcheri*.

2. **Cannibalism Study:** To investigate cannibalism as a means of mite-to-mite transmission.

3. **Mite Colonization:** To continue investigations of vector bionomics and to maintain material for attempted infection, rickettsial strain stability and other studies.

4. **Vector Distribution:** To determine if *L. (L.) arenicola* or other vectors found in beach scrub in West Malaysia are present in similar habitats in Indonesia.

5. **Ecological Studies:** To study populations of scrub typhus vectors in a variety of adjacent habitats to determine the effects of seasonality and other factors.

6. **Miscellaneous Collections:** To continue surveys of scrub typhus vectors in Malaysia.

Studies of *Rickettsia tsutsugamushi*

1. To study the various effects of scrub typhus in primates:

    (a) Development of the silvered leaf-monkey as a primate model for scrub typhus.

    (b) Dose and strain response.

    (c) Rechallenge studies.

(d) The relationship between strain, dose and eschar formation in silvered leaf-monkeys.

(e) Gibbon studies including therapy and rechallenge.

2. To determine if *R. tsutsugamushi* remains antigenically stable in:

(a) In a primate, the silvered leaf-monkey, as a model for human disease.

(b) Vector *Leptotrombidium* mites using material from the positive colony.

(c) In wild rodents, *Rattus annandalei*.

3. To infect Leptotrombidium mites with known characterized strains of *R. tsutsugamushi*.

4. To study the effects of freezing and storage temperatures on maintenance of high suspensions titers of *R. tsutsugamushi*.

5. To initiate a long term study on the effect of differences in seasons and habitats on rickettsial isolation and serology ratios in small mammals.

6. To initiate a study of the effect of the immune or resistance status of rodent host on the antigenic stability and virulence of *R. tsutsugamushi* in Leptotrombidium mites using the positive *L. (L.) fletcheri* colony.

Studies of Scrub Typhus Vectors

Microdissection of Infectious Mites:

General: The microdissection technique (Annual Report 1971) and direct fluorescent antibody test were used to study the distribution of *R. tsutsugamushi* in tissues from each life stage of infectious (positive) laboratory-reared *L. (L.) fletcheri*.

Methods: All mites dissected were offspring from known positive females. Initially, mites were incubated in humidified rearing containers at 34°C for 48 hours before dissection in an attempt to increase rickettsial concentration. But since no differences in incubated and non-incubated concentrations were observed, incubation was discontinued. The individual tissue to be examined was dissected out, and 3 spots from the "center" of the dissected material were made on a slide. After fixing with acetone, 2 of the spots were stained with a fluorescein isothiocyanate conjugate of the Karp and Kato strains of *Rickettsia tsutsugamushi* supplied by WRAIR. The third spot was used as a control and was stained with a normal rabbit serum conjugate. A Rhodamine counterstain was used to increase contrast.

Results: The per cent of tissues containing rickettsia in each
life stage are shown in Table 1. Rickettsia were present in the gut and
hemolymph of all stages examined. Engorged larvae had a notably lower
percentage of tissues with rickettsia than did the other stages, but
unengorged larvae had the highest percentage of tissues positive.
Engorged larvae were much more difficult to dissect, and dilution by the
ingested fluid may have also contributed to the lower percentage. Both
adult and nymphal stages had a much higher rate of positive ovarian
tissue than did the teliophane stage. The considerable metamorphosis
tnat occurs in the teliophane may be associated with this difference.
The teliophane stage had the highest percentage of excretory tubule
tissue with rickettsia. Contents of 12 eggs from 4 positive *L. (L.)
fletcheri* female were also examined, and 11 (91.7%) were positive. This
percentage is close to that determined by Rapmund *et al* (1969) as the
rate of transovarial transmission. Fluorescent antibody examination of
eggs appears to be a feasible means for screening field collected vectors,
for colonization, since the adult is kept alive. Hemolymph screening is
possible but less desirable, since mortality is high. For screening
potential vectors when colonization is not an objective, gut and
hemolymph appear to be the tissues of choice.

Cannibalism Study: The possibility of cannibalism as a means of
mite-to-mite transmission of *R. tsutsugamushi* was considered following
the observation of nymphal mites feeding on prenymphs of the same species
(Annual Report 1971). However, the one line of mites that appeared to be
infected following cannibalism (13/15 offspring from a single female) did
not survive. A total of 38 other attempts were made with non-infectious
(negative) nymphs of all 3 colonized vector species, but none have
produced infectious offspring. Eggs from positive females have also been
fed to the negative nymphs. Results of some of the mouse passages are
still pending, but if cannibalism results in mite-to-mite transmission,
the rate of this transmission is probably very low.

Mite Colonization: Colonies of the known vectors *L. (L.) arenicola,
L. (L.) fletcheri* (= *akamushi*), and *L. (L.) deliense* are being maintained
for attempted vector infection, virulence and rickettsial strain stability
studies and for bionomic and behavioral observations. Other colonies of
L. (L.) bodense and *Blankaartia ascoscutellaris* are also being maintained.
These 2 species are not known to be vectors but have been used to sensitize
mice for attempted infections. As potential vectors are collected, they
will be colonized for testing.

New collections of *L. (L.) arenicola* were made 23-25 March from beach
scrub areas near Mersing on the Southeast coast of West Malaysia.
Approximately 2000 engorged chiggers were collected from rodents, and
offspring will be screened by direct FA. Subsequent individual mouse
feedings and passages will be made with other offspring from any females
thought to be infectious by FA examinations.

The positive colony of *L. (L.) fletcheri* is into the eleventh
laboratory generation, but egg production has declined below that of the
non-infectious colony since December 1971. No apparent differences
exist in rearing or handling except that the positive mites are maintained

Table 1

Distribution of *Rickettsia tsutsugamushi* in Tissues of Infectious Laboratory-reared
Leptotrombidium (L.) akamushi

TISSUE	Larvae				Nymphs		Teliophanes		Adults	
	unengorged		engorged							
	%+	(total)	%+	(total)	%+	(total)	%+	(total)	%+	(total)
Salivary gland	78%	(9)	0%	(6)	50%	(6)	70%	(10)	38%	(8)
Gut	89%	(9)	17%	(6)	50%	(6)	44%	(9)	40%	(10)
Excretory tubule	*		0%	(3)	0%	(3)	71%	(7)	29%	(7)
Epidermal	*		0%	(4)	50%	(8)	30%	(10)	50%	(8)
Ovary	*		*		78%	(9)	14%	(7)	73%	(11)
Hemolymph	75%	(8)	40%	(5)	38%	(8)	67%	(6)	50%	(6)
% tissues positive at each life stage	81%		13%		50%		49%		48%	

* Tissue undeveloped or too small for determination.

individually, while the negative colony is maintained in pools. Further, males from the non-infectious colony are constantly introduced into the positive colony. The observation by Rapmund *et al* (1969) that a positive female from this colony has never produced a male offspring remains valid. Previously, average egg production was 141.9 for the positive colony and 118.3 for the negative colony. The rates of hatch were almost identical: 75.5% for the negative colony and 76% for the positive colony. In an effort to increase egg production in the positive colony, field-collected males are being introduced. Large numbers of infectious mites have been used over the past year, and the colony may have been overly "pressured".

The female to male ratio of the negative colony was 2.45:1, based on 1589 progeny over 4 generations. In over 11,000 offspring, no males have been produced by known infectious females from the positive colony. As stated in the 1971 Annual Report, isolated, uninseminated females from the negative colony failed to produce offspring. Thus, "normal" thelyotoky (parthenogenetic reproduction in which the progeny are all females) would not explain the lack of males. However, some species of mites are gynogenetic-must take up sperm to reproduce parthenogenetically, even though the eggs are not fertilized (Oliver 1971). Thelyotokus gynogenesis could result in only female offspring, but cytological evaluation will be necessary to determine if this form of reproduction is occurring in the positive colony.

Vector Distribution: In West Malaysia, the sandy beach areas shaded by scrub vegetation and occasional secondary trees form the typical habitat of *L. (L.) arenicola* Traub. Previous studies have mapped the distribution of this vector in West Malaysia (Upham *et al*, 1971). But in Sabah and Sarawak in East Malaysia and along the beaches of Southern Thailand, another vector, *L. (L.) deliense* was found in beach scrub habitats. In attempt to further define the distribution of scrub typhus vectors in coastal habitats of Southeast Asia, studies were made of beach habitats in Java and North Sumatra, Indonesia in cooperation with the Navy Medical Research Unit II, Djakarta.

Methods: Rodent trapping and black plate surveys were made in beach scrub habitats near Labuan, West Java and between the coastal villages of Tandjung Tiram and Telukpijai on the west coast of North Sumatra. Blood samples were taken from rodents and spotted on filter paper strips for subsequent IFAT examination.

Results: West Java - Thirty-five rodents were captured from 4-7 September (Table 2) but none were infested with known vectors of scrub typhus. *Ascoschoengastia (Laurentella) indica* was the most commonly collected chigger species. Gamasoid mites were collected from 9 rodents. One filter paper blood sample from a *Rattus r. diardii* was positive for *R. tsutsugamushi*. No known vectors were collected in beach habitats by black plating. Based on Malaysian collections, the weather was too dry for favorable black plate collections.

Table 2

Gamasoid Mites and Chiggers Collected from Rodents Captured
near Labuahn, West Java Coast, September 1971.

Host Species	No. Collected	Gamasoid Mites			Chigger Species	
		Hirstionyssus spp.	*Haemolaelaps spp.*	*Laelaps spp.*	*A. (L.) indica*	*Eutrombicula wichmanni*
Callosciurus notatus	6	17	22	–	–	10
Chiropodomys gliroides	1	–	–	4	–	–
Hylopetes spadiceus	1	–	–	3	–	–
Petaurista petaurista	1	–	2	–	–	–
Rattus r. diardii	26	–	–	–	131	–

North Sumatra - From 31 May to 2 June, 27 rodents were trapped from beach scrub, and 15 had chiggers attached (Table 3). One *R. r. argentiventer* had 31 *L. (L.) deliense* attached, but again the most common chigger species collected was *A. (L.) indica*. No known vectors were collected by black plating.

Although collections in Indonesia were quite limited, *L. (L.) deliense* was the only vector collected. Habitats appeared to be very similar to those of coastal West Malaysia where *L. (L.) arenicola* is common, but this species was not collected. More extensive collections would be necessary to confirm the presence or absence of *L. (L.) arenicola* in Indonesia.

Ecological Studies of Scrub Typhus Vectors: A study of vector populations in a variety of adjacent habitats was started at Bukit Lanjan, Selangor in August 1971 and will continue through October 1972. The study is being coordinated with the Departments of Medical Ecology and Rickettsiology. The area (see Figure 2, Medical Ecology Section) is located about 10 miles Northwest of Kuala Lumpur and includes lalang grass, scrub, an aboriginal village with surrounding scrub vegetation and disturbed primary forest. The lalang habitat consists mainly of *Imperata cylindrica* with few shrubs, vines or other scrub plants. It is about 100 meters wide and extends to the horizon beneath an electrical power line. The outer secondary forest fringe or scrub consists of an undergrowth of vines, broad leaf plants, shrubs and young trees. The forest habitat is made up of long-standing secondary trees with tracts of primary forest trees of the dipterocarp family, and the forest floor has a thick layer of humis.

Specific Acarology objectives were to: (1) take interval samples of the populations of vector species present in each habitat to determine when and why fluctuations in numbers occur; (2) compare rodents from each habitat in terms of numbers of chiggers attached; (3) determine if there is a correlation between isolation rates of scrub typhus in rodents and numbers of vectors collected; (4) determine what other chigger species are present and if they could be scrub typhus vectors.

Methods: Chiggers were collected by both rodent trapping and the black plate method. Thirty-two mite foci (8 per habitat) were located by preliminary black plate survey and marked with a wooden stake. At each focus, 10 black plates were placed within a 2 feet square area. Unengorged chiggers were collected 3 times per week through January 1972 and twice per week thereafter. Microhabitat temperature readings were taken with an electric thermometer, and a hygrothermograph at ground level was used to measure air temperature and relative humidity. Rainfall data was collected at the nearby Subang weather station.

After 1 month of preliminary trapping, 4 representative rodent species were selected for monitoring: *Rattus sabanus*, a forest rat; *R. argentiventer* and *R. tiomanicus jalorensis*, captured mostly in lalang and scrub at Bukit Lanjan; *R. exulans*, usually trapped in and

Table 3

Larval Mites Collected from Rodents Captured in Beach Scrub
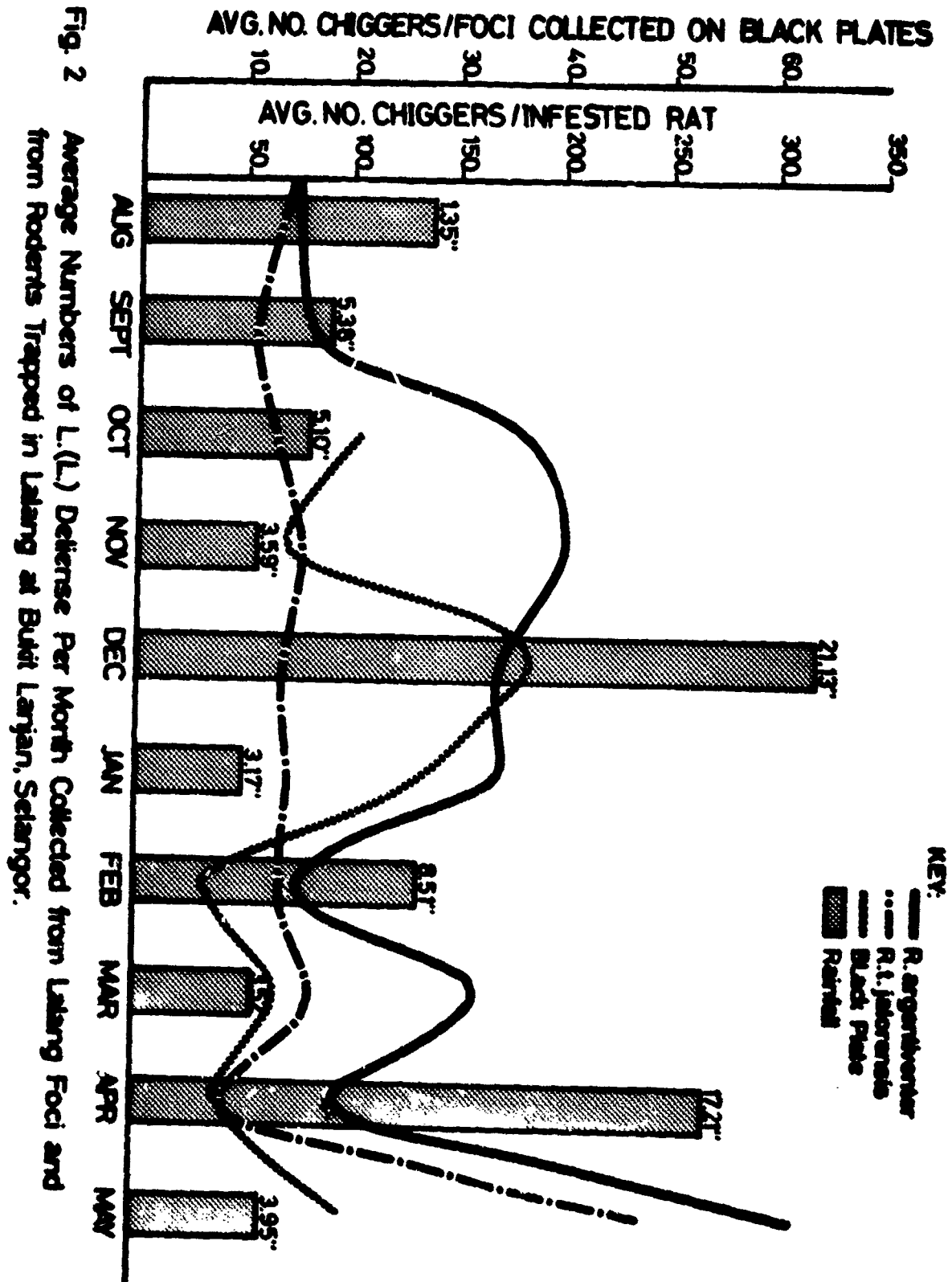between Tandjung: Tiram and Telukpijai, North Sumatra,
31 May to 2 June, 1972

Host, Species	No. with No. Chiggers/Examined	Chigger Species	No. Present
Rattus argentiventer	5/9	*Aschoschoengastia (Lau.) indica*	243
		Leptotrombidium (L.) deliense	31
R. r. diardii	9/13	*A. (L.) indica*	671
R. exulans	0/1	-	-
R. tiomanicus jalorensis	0/2	-	-
R. whiteheadi	1/1	*A. (L.) indica*	1
Callosciurus notatus	0/1	-	-
Total	15/27		946

around the aboriginal houses. Rodents were trapped 5 days per week,
brought to the laboratory and anesthetized so that chiggers and blood
samples could be collected. After marking by toe clipping, the
rodents were released into the same habitats in which they were
captured.

Results: The only vector species collected on black plates
was *L. (L.) deliense.* Average weekly numbers of chiggers per black
plate were determined for each habitat and compared to rainfall data.
Comparisons of chigger numbers with atmospheric moisture and
temperature are in progress. Results of collections through the
first 14 weeks are shown in Figure 1. It appeared that the average
weekly rainfall could have had a delayed effect, with numbers of
chiggers increasing following an increase in rainfall during the
preceding week. Sample trends were generally similar in all
habitats during the first 9 weeks, with a decline in numbers
occurring during weeks 2-6. This coincided with a general decrease
in average rainfall. Numbers from lalang and scrub remained higher
than those from village scrub and primary forest during weeks 10 to
14 and seemed more closely related to average rainfall. During
January, a 3 week logging operation in the primary forest area and
resulting disturbance in the nearby scrub interrupted the black plate
study of these habitats. The village scrub area and lalang were
considered to be far enough from the logging to permit continued black
plate collections. Primary forest rodent trapping was shifted to a
second primary forest tract located about 900 meters North of the
original site. Trapping in the scrub area was halted until after
logging was completed and then was continued in the same general
location. Vegetation in the scrub trapping area was not disturbed
by the logging. Figure 2 shows the average monthly chigger numbers
by black plate sampling of lalang foci and the average numbers from
infested rodents trapped in lalang compared to average monthly
rainfall. No consistent correlation could be made between chigger
numbers and average monthly rainfall. The foci collections and those
from *R. argentiventer* trapped in lalang were similar from December
through May. *R. t. jalorensis* trapped in lalang had notably fewer
chiggers than *R. argentiventer*, but 399 infested *jalorensis* were
captured or recaptured from lalang while only 129 infested
argentiventer were captured or recaptured during 10 months of study.
To date, the highest numbers of chiggers on these 2 rodent species
have occurred during May, but highest numbers on black plates were
collected during December. The average numbers of chiggers per
scrub foci and the averages per infested rat trapped in scrub were
much less similar than the averages in lalang (Figure 3). The
greater variability of the scrub habitat probably contributed to the
differences. No consistent relationship between rainfall and chigger
numbers was apparent. Except for January, *R. argentiventer* had
consistently higher numbers of chiggers than did *R. t. jalorensis.* Total
captures and recaptures yielded 488 (95.7%) infested *jalorensis* and 136
(98.5%) infested *argentiventer.* It was found that male rats of both species

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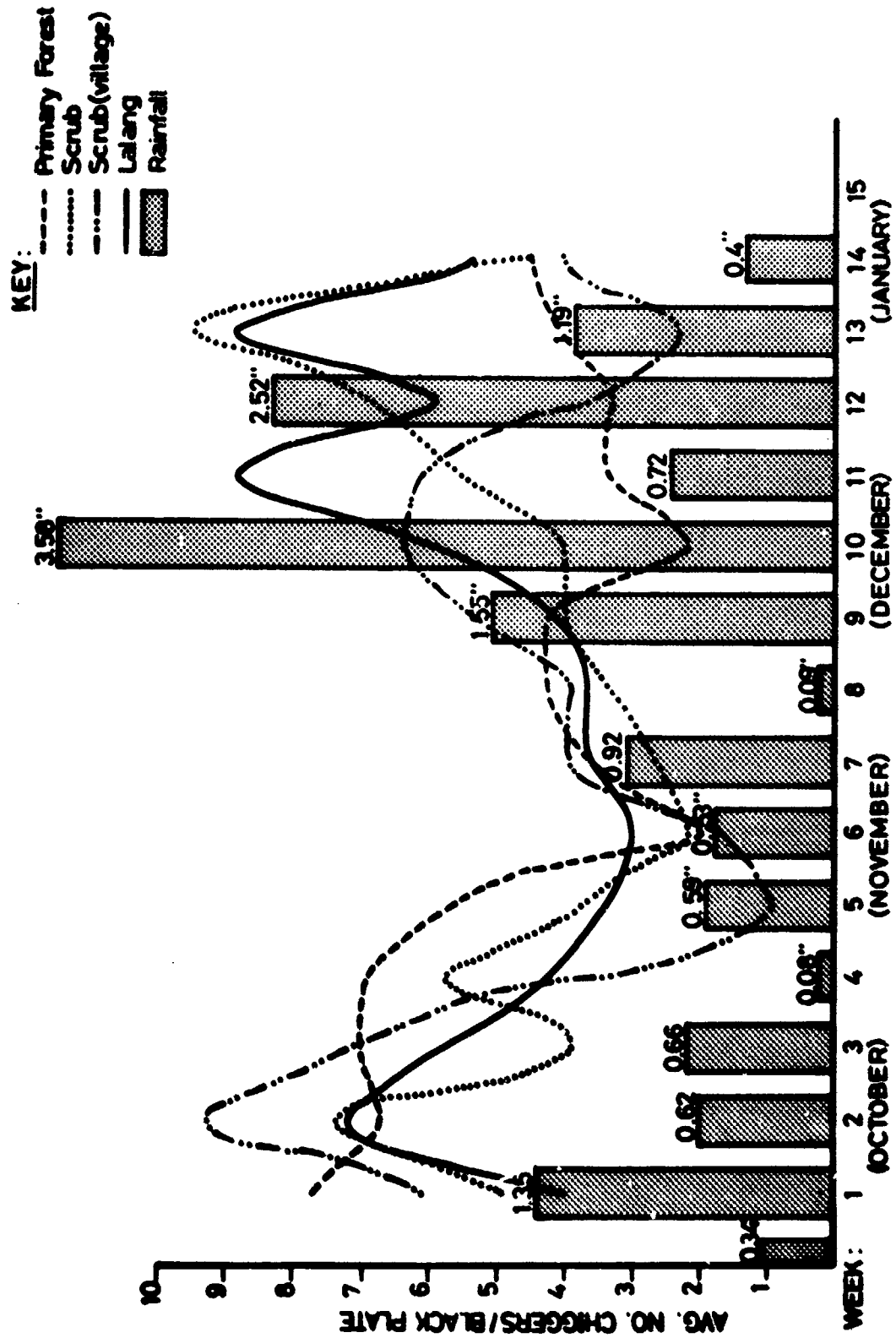
Fig. 1 Black Plate Collections of Leptotrombidium(L) deliense from Four Habitats at Bukit Lanjan, Selangor, 4 October 71 to 7 January 72.

Fig. 2 Average Numbers of L.(L.) Deliense Per Month Collected from Leleng Foci and from Rodents Trapped in Leleng at Bukit Lanjan, Selangor.
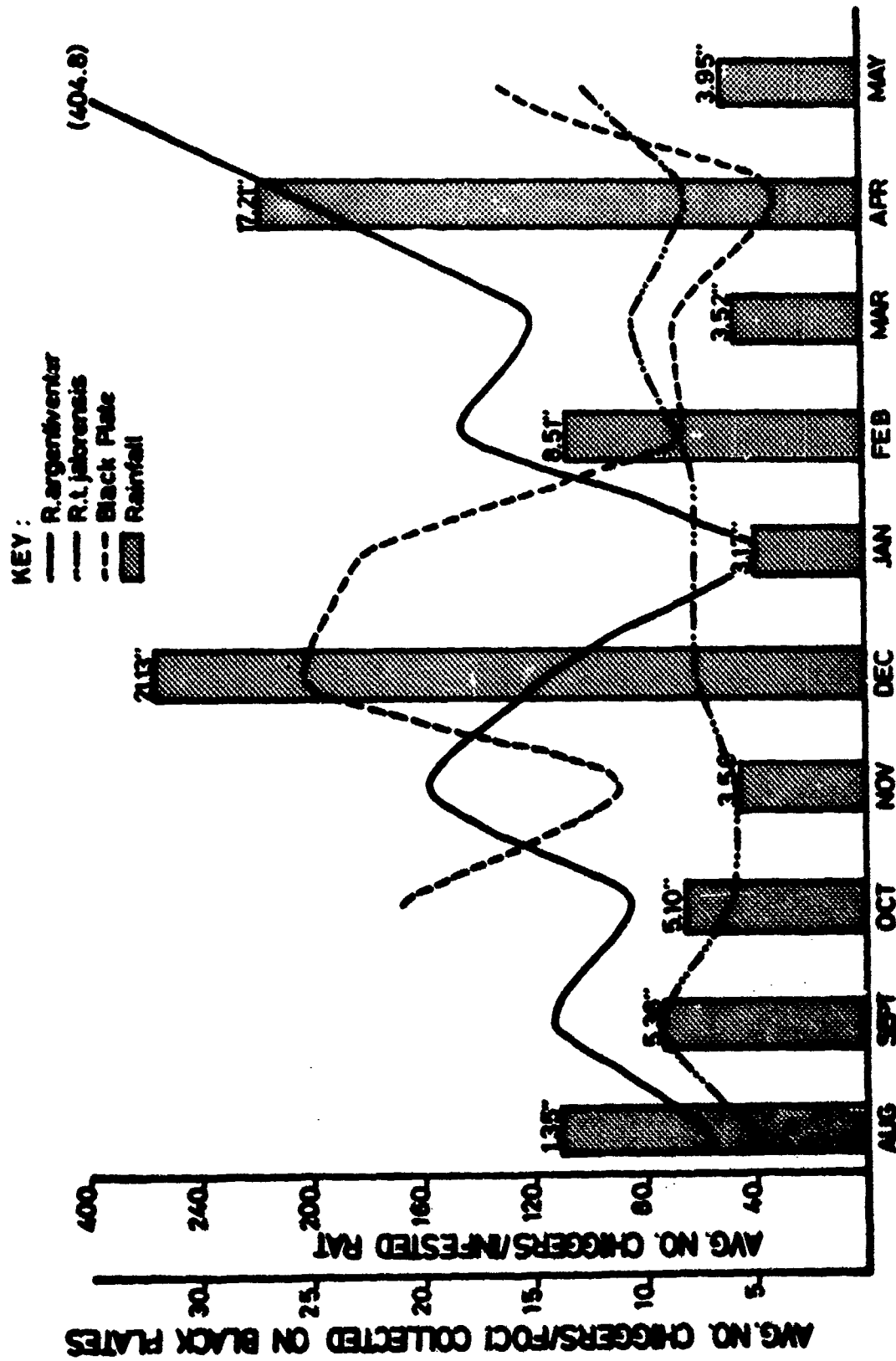
Fig. 3 Average Numbers of L.(L) Deliense per Month Collected from Scrub Foci and Rodents Trapped in Scrub at Bukit Lanjan, Selangor.

trapped in both lalang and scrub usually had higher average numbers of chiggers than did females (Table 4). The majority of the noninfested rats of both species have been female: 75% of the noninfested *argentiventer* and 67.5% of the noninfested *jalorensis* were females.

A commensal rat, *R. exulans* averaged only 7.5 chiggers per infested rat, and 62% of the 354 captured or recaptured rats examined were noninfested. A single individual had 140 chiggers. *Rattus sabanus* was the only host to be consistently infested with another species of chigger. *Gahrliepia fletcheri* was more abundant than *L. (L.) deliense* on *R. sabanus* for every month except February (Figure 4). Again, increases in *deliense* numbers did not consistently correspond with increases in rainfall. The average numbers of *G. fletcheri* were relatively constant from November to January and from March through May.

Isolation data are not yet available for comparison with chigger numbers. Two black plate foci, one in the village scrub and one in lalang, have yielded *L. (L.) deliense* larvae that were positive for rickettsia by the indirect fluorescent antibody test. The vector *L. (L.) fletcheri (=akamushi)* has not been collected although the lalang appears favorable for this species. A total of 16 other species were collected, but with the exception of *G. fletcheri*, numbers have been too low for study.

Discussion: Since the study is still in p. ogress, all considerations are preliminary. Although average weekly black plate numbers in lalang and scrub appear to correlate with average weekly rainfall, it does not appear that the average monthly *L. (L.) deliense* population at Bukit Lanjan can be consistently correlated with rainfall alone. Previous workers have stated that *deliense* populations fluctuate with rainfall, but data to demonstrate this are generally lacking. It seems likely that a complex interaction of factors such as the temperature and moisture level of the microhabitat, host activities, availability of adult food may be of importance in addition to rainfall. Further comparisons are planned when more data are available.

While similarities existed between populations at the various foci and those on rodents, marked differences did occur. In a vector control program, the method of sampling used could be quite important in determining times and methods for treatment. *R. argentiventer* was the most important host in terms of numbers of chiggers per infested rat, but all hosts will be reevaluated when isolation results are complete. The absence of *L. (L.) fletcheri* from the lalang habitat is somewhat surprising, since the lalang field has been in existence for over 10 years and is continuous with the power line. No other species collected to date appear to be likely vectors.
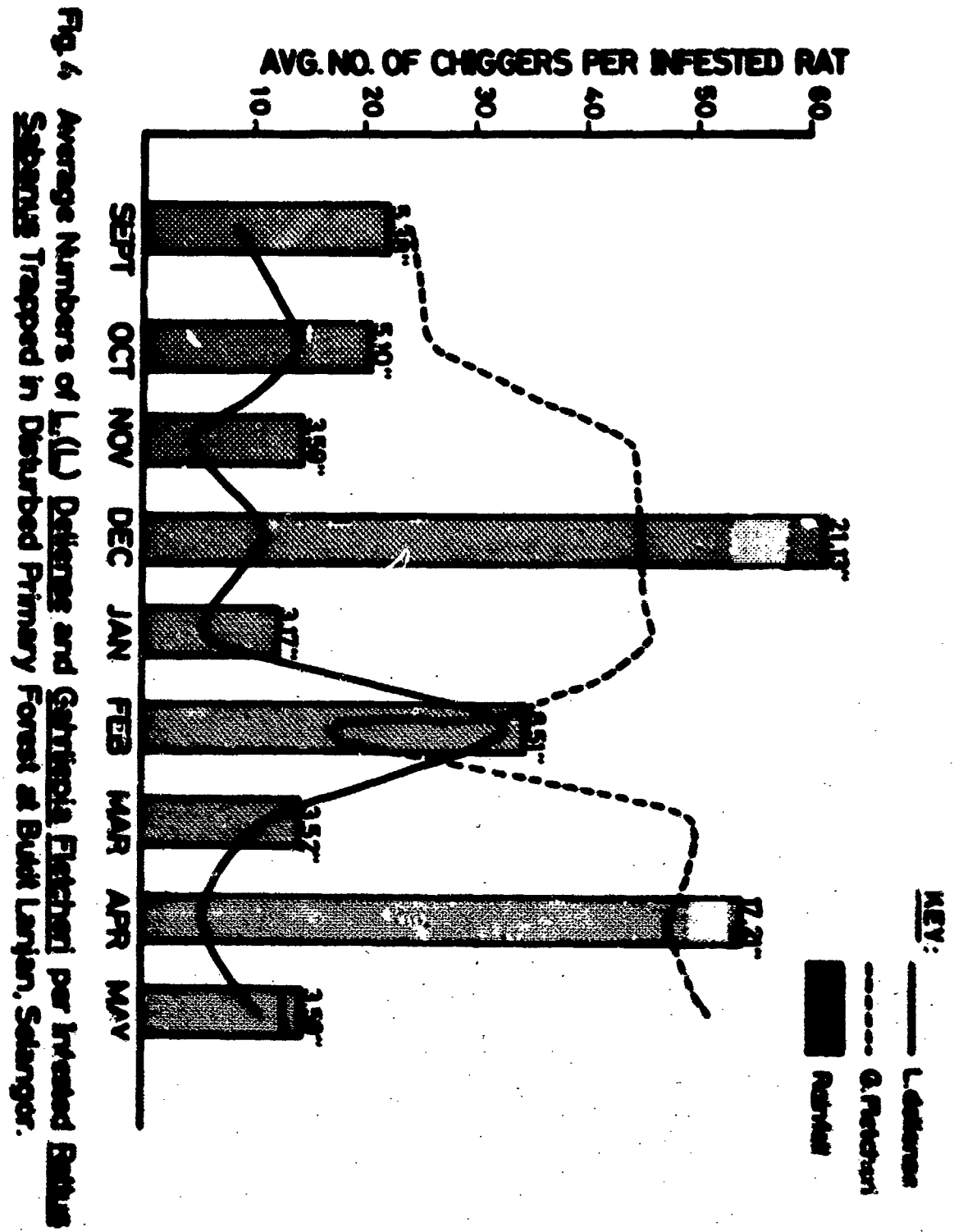
Miscellaneous Collections: Chiggers were collected from mammals trapped by the Department of Medical Ecology in Ranau, Sabah, East Malaysia. The mammals were taken from a slightly disturbed primary

Table 4

Chigger Averages by Sex of the Hosts: Infested *Rattus tiomanicus jalorensis* and *R. argentiventer* trapped in Lalang and scrub at Bukit Lanjan, Selangor.

Month	R. t. jalorensis				R. argentiventer			
	Lalang		Scrub		Lalang		Scrub	
	Females	Males	Females	Males	Females	Males	Females	Males
August	72.3	67.3	41.8	39.3	58.3	87.3	17.5	-
September	42.8	61.5	85.2	67.9	55.3	93.4	105.3	126.3
October	52.2	77.7	31.7	79.7	194.8	156.6	61.3	150.6
November	98.6	72.2	32.2	83.6	136.3	275.3	180.0	153.1
December	61.0	76.6	42.0	91.8	168.9	177.4	93.1	130.6
January	52.1	86.5	50.0	70.8	115.2	213.3	31.0	53.3
February	39.1	86.8	55.2	87.6	86.2	68.6	144.4	160.4
March	85.0	88.0	63.8	112.4	197.6	142.0	93.4	146.9
April	32.8	49.8	58.2	78.9	-	95.8	169.7	206.5
May	46.9	123.0	101.3	105.5	-	313.0	486.9	299.6
Average	58.3	79.4	56.1	82.7	126.6	162.3	142.3	158.6

Fig. 6 Average Numbers of L.(L) Deliense and Gahrliepia Fletcheri per Infested Rattus Sabanus Trapped in Disturbed Primary Forest at Bukit Lanjan, Selangor.

forest habitat within the Kinabalu National Park and from secondary forest near the village of Nalapak. The last previous chigger collections from the Mt. Kinabalu area were made by Traub and Audy, June 1952.

Thirty-one mammals were trapped from 1-4 June 1971 in the North Park forest, and 703 chiggers were collected (Table 5). The most abundant chigger species was *Walchiella oudemansi*, representing 49.6% of the total. The vector *L. (L.) deliense* was collected in low numbers from 3 host species.

Only 7 mammals were collected from the secondary forest area. *L. (L.) deliense* was collected from 4 of these (Table 6). A primate, *Tarsius bancanus*, had 83 *deliense* attached.

Studies of *Rickettsia tsutsugamushi*

Primate Studies:

General: Preliminary data on the susceptibility of the silvered leaf-monkey to *R. tsutsugamushi* has been reported in the last two annual reports (USAMRU, Malaysia, Annual Report 1970 and 1971). All of previous work was done either with the Karp strain of *R. tsutsugamushi* or an untyped human isolate. During this reporting period these studies were expanded to determine the response of animals inoculated with different prototype strains (see WRAIR Annual Report 1971). In the first group of animals, the strains were mixed, but in the remaining animals they were always inoculated in separate sites.

Materials and Methods: The serologic test employed in these studies was the indirect fluorescent antibody test developed by the Department of Rickettsial Diseases, WRAIR. The antigen was composed of the Karp, Gilliam and Kato strains of *R. tsutsugamushi*.

All challenge strains and serologic antigens were supplied by the Department of Rickettsial Diseases, Walter Reed Army Institute of Research, Washington, D.C. Standard isolation and titration techniques in mice were utilized throughout, employing the intraperitoneal route (IP) (USAMRU, Malaysia, Annual Report 1971). The WCI mice were from the animal production unit of the Institute of Medical Research, Kuala Lumpur, Malaysia. Rickettsemias were determined by injecting five mice IP with 0.2 ml of whole blood diluted 1:10 in chilled Snyder's buffer. From this 1:10 dilution of blood, serial 10 fold dilutions were made to determine the level of rickettsemia per ml of whole blood. The same technique was used in organ titrations except that the original 1:10 dilution was calculated according to weight/volume and the organs were ground using a hand operated glass tissue grinder. To confirm that isolates were *R. tsutsugamushi*, organ pools from the first-passage mice from two different days of rickettsemia and from two organs for each monkey were passed into 10 second-passage

Table 5

Chiggers Collected from Mammals Trapped in North Park,
Ranau, Sabah, 1-4 June 1971

Chigger Species	Host (No. Trapped)	*Sundasciurus lowii* (1)	*Rattus cremoreventer* (3)	*R. muelleri* (12)	*R. whiteheadi* (6)	*Tupaia ta'a* (9)	Total (31)
Chelodenta susa reidi		-	-	7	-	-	7
Eutrombicula wichmanni		-	-	4	-	-	4
Gahrliepia (W.) disp. disparunguis		-	-	14	164	3	181
Helenicula signata		-	-	-	-	111	111
Leptotrombidium (L.) deliense		-	3	9	-	39	51
Walchiella oudemansi		15	4	149	-	185	353

Table 6

Chiggers Collected from Mammals Trapped in Secondary Forest
near Nalapak, Ranau, Sabah, 4-6 June 1971

Chigger Species	*Callosciurus notatus* (1)	*Hylopetes spadiceus* (1)	*Rattus r. muelleri* (2)	*R. r. whiteheadi* (1)	*Tarsius bancanus* (1)	*Tupaia tana* (1)	Total
Gahrliepia (W.) disp. disparunguis	-	-	8	12	-	-	20
Eutrombicula wichmanni	8	-	-	-	-	1	9
Helenicula signata	-	-	1	-	-	16	17
Leptotrombidium (L.) deliense	3	1	1	-	83	-	88
Walchiella impar	-	8	-	-	-	-	8
W. oudemansi	13	-	-	-	-	2	15

mice. These second-passage mice were challenged at 28-32 days post-inoculation with 10^{3-4} MIPLD$_{50}$'s of the mouse-lethal Karp strain of *R. tsutsugamushi*.

Results and Discussion of the First Multistrain Challenges and Serological Results: Serologic testing for scrub typhus revealed that 44 per cent of the 75 cynomolgus monkeys, *(Macaca fascicularis)*, and 36 per cent of the 75 pig-tailed monkeys, *(Macaca nemestrina)* from Malaysia were positive at a 1:40 dilution or higher. Of the 75 silvered leaf-monkeys, *(Presbytis cristatus)* tested, none were positive. In addition to those with a positive titer, one can assume that a large percentage of those negative at a 1:40 dilution of serum has some degree of resistance. Further, since one can presume that this exposure is not something new, one assumes that there has been a steady selection toward animals more resistant to the effects of infection. It also supports our supposition that the silvered leaf-monkeys would be susceptible because of their mangrove swamp habitat and arboreal habits. These animals represent our preliminary work to determine whether they are susceptible to scrub typhus. Extensive laboratory work on a daily basis was not performed and in some cases strains of *R. tsutsugamushi* were mixed to obtain information as to whether multistrain challenge would make the infection more acute and/or severe. Small numbers of animals were utilized for certain strains to obtain the maximum amount of information with a limited number of animals. Therefore the data in Table 7 are presented in two groups in order to have an adequate number of animals in each group. Later studies are more detailed and involve larger groups of animals. Table 7 represents the preliminary data on 14 silvered leaf-monkeys. During the height of illness, which occurred between two and four weeks post-challenge, the animals became very weak and could be handled almost as if they were tame. Many of them became so weak that they could no longer sit on their perches but went to the floor of the cage to sit--something they never did when healthy. All animals developed a hyperthermia; however, not all of the hyperthermias were prolonged for more than five days. It appears from the data given in Table 7 that the Gilliam strain produced a more prolonged hyperthermia than did the Karp or Kato strains. The same was true of the eschars, in that while all animals developed eschars which appeared grossly to be identical with those found in humans, the eschars produced by the Gilliam strain lasted longer than those produced by Karp or Kato alone. The mean day of onset of rickettsemia was strain dependent. Those animals receiving Gilliam either singly or in combination had rickettsemia beginning on day 4, (range 2 to 5), while those receiving only Karp or Kato had rickettsemia begin on day 6, (range 3 to 8). The average duration of rickettsemia was 16 days for all strains and the range of titers was from 10^1 to 10^3 MIPLD$_{50}$'s per ml of whole blood. Some of the mice were checked for ID$_{50}$'s as well; however, this was discontinued when it was observed that rarely was there over $10^{0.5}$ difference between the ID$_{50}$ and LD$_{50}$.

It was desirable to determine whether or not silvered leaf-monkeys could become chronically infected (Table 8). Almost all

Table 7

The Response of Silvered Leaf-monkeys to Intradermal Inoculation with *R. tsutsugamushi*

Parameters	Karp or Kato[1]	STRAINS Gilliam or a mixture[2] containing Gilliam	Total
Clinical illness	$8/8^3$	6/6	14/14
Eschar formation	8/8	6/6	14/14
Eschar lasting 15 da. or longer	0/8	6/6	6/14
Hyperthermia (5 da $\geq 103^{\circ}F$)	3/8	5/6	8/14
Rickettsemia (POS AT 10^{-1} of whole blood)	8/8	6/6	14/14
Chronic infection in survivors	6/7	5/5	11/12
Serologic conversion in survivors (titer of 1:40 or greater)	7/7	5/5	12/12
Death	$1/8^4$	$1/6^5$	2/14

1. Karp and Kato; 5 animals received the Karp strain,
    1 animal received the Kato strain,
    2 animals received both Karp and Kato.

2. Gilliam or a mixture containing Gilliam;
    1 animal received the Gilliam strain,
    1 animal received both Gilliam and Kato,
    2 animals received both Gilliam and Karp,
    2 animals received all three, Gilliam, Karp and Kato.

3. Number responding over total number inoculated.

4. The animal died on day 29 post-inoculation and received Karp.

5. The animal died on day 20 post-inoculation and received Karp, Gilliam and Kato.

Dosages: Karp $10^{4.4}$ MIPLD$_{50}$; Kato $10^{3.7}$ MIPLD$_{50}$; Gilliam $10^{4.0}$ MIPID$_{50}$

Table 8

Isolation of *Rickettsia tsutsugamushi* from Tissues Two Months Following Inoculation

ORGANS	STRAINS				TOTAL
	Karp or Kato[1]		Gilliam or a mixture containing Gilliam[1]		# Positive/Total #
	# Positive/ Total #	MIPLD$_{50}$'s per gram or ml	# Positive/ Total #	MIPLD$_{50}$'s per gram or ml	
Blood	0/7	-	1/5	2.0	1/12
Liver	1/7	1.6	0/5	-	1/12
Lung	0/7	-	1/5	2.0	1/12
Kidneys	1/7	1.6	2/5	1.8	3/12
Spleens	2/7	1.6	2/5	1.8	4/12
Axillary lymph nodes	6/7	3.2	5/5	3.1	11/12
Inguinal lymph nodes	6/7	3.4	5/5	3.1	11/12
Titer, IFAT, median titer	1:160		1:160		

1 Challenges and dosages same as Table 7.

animals become chronically infected and the lymphatic system is the
system most often involved. Not only were the lymph nodes draining the
site of inoculation positive in 11/12 cases (inguinal lymph nodes),
but also the axillary lymph nodes. The lymph nodes also had a higher
titer per gram of tissue than any of the other organs. It appears
that one of the animals receiving Gilliam had a rickettsemia on day 60
when it was euthanized. We have observed a death as late as 63 days
post-inoculation in one of the silvered leaf-monkey challenged with
Karp and Gilliam. In the case of two silvered leaf-monkeys checked
for chronic infection at five months, *R. tsutsugamushi* was isolated
from the axillary and inguinal lymph nodes. The peak of clinical
illness in silvered leaf-monkeys occurs between three and four weeks
post-inoculation while in humans it occurs in the second and third
weeks of illness (which is the third and fourth week post-infection).
All animals developed eschars at the site of inoculation with Karp,
Kato and Gilliam strains. In summary, it appears that the illness
produced in silvered leaf-monkeys following an intradermal challenge
with *R. tsutsugamushi* is remarkably similar to that seen in man and
that the silvered leaf-monkey can be utilized as a subhuman primate
model for human scrub typhus.

*Dose Response in Silvered Leaf-monkeys to the Karp and
Gilliam Strains:* To further elucidate the susceptibility of the
silvered leaf-monkey and determine the dynamics of the infection, a
group of 20 animals were challenged with varying doses of the Karp and
Gilliam strains inoculated in separate sites on the thighs. Figure
5 gives the data obtained. The lines represent the mean values for
5 animals at each dose. The following parameters are dose dependent:
Onset of rickettsemia, death, temperature and white blood cell
response. The packed cell volume appears not to be dose dependent
but is drastically lowered in all cases. The animals developed
first a hyper- and then a hypothermia which reached its low point
between 20 to 25 days post-inoculation discounting the animals which
had received a dose of 10^8 organisms and which died before that time.
The eight animals that survived ran temperatures less than 100°F for
several days (normal temperature 102°F). The maximum WBC response and
drop in PCV occurred between 20 and 26 days. The LD_{50} in this group
of animals was $10^{6.8}$ $MIPLD_{50}$'s. The time that it takes eschars to
form and their formation is dose dependent and the duration is strain
dependent, according to Table 9. At a dose of $10^{2.1}$ Karp failed to
produce any eschars in 5 monkeys while the same animals produced
Gilliam eschars at a dose of $10^{1.5}$ $MIPID_{50}$. Two animals had Gilliam
eschars at 30 days and one animal still had a Gilliam eschar at 42
days.

Infection of Silvered Leaf-monkeys for Pathology Specimens:
Since little is known of the pathology of scrub typhus except that
obtained from human fatalities and recognizing that knowledge of the
pathology of scrub typhus would be necessary for any vaccine trials
conducted using silvered leaf-monkeys, specimens for pathologic
studies were collected. In addition, it was necessary to determine
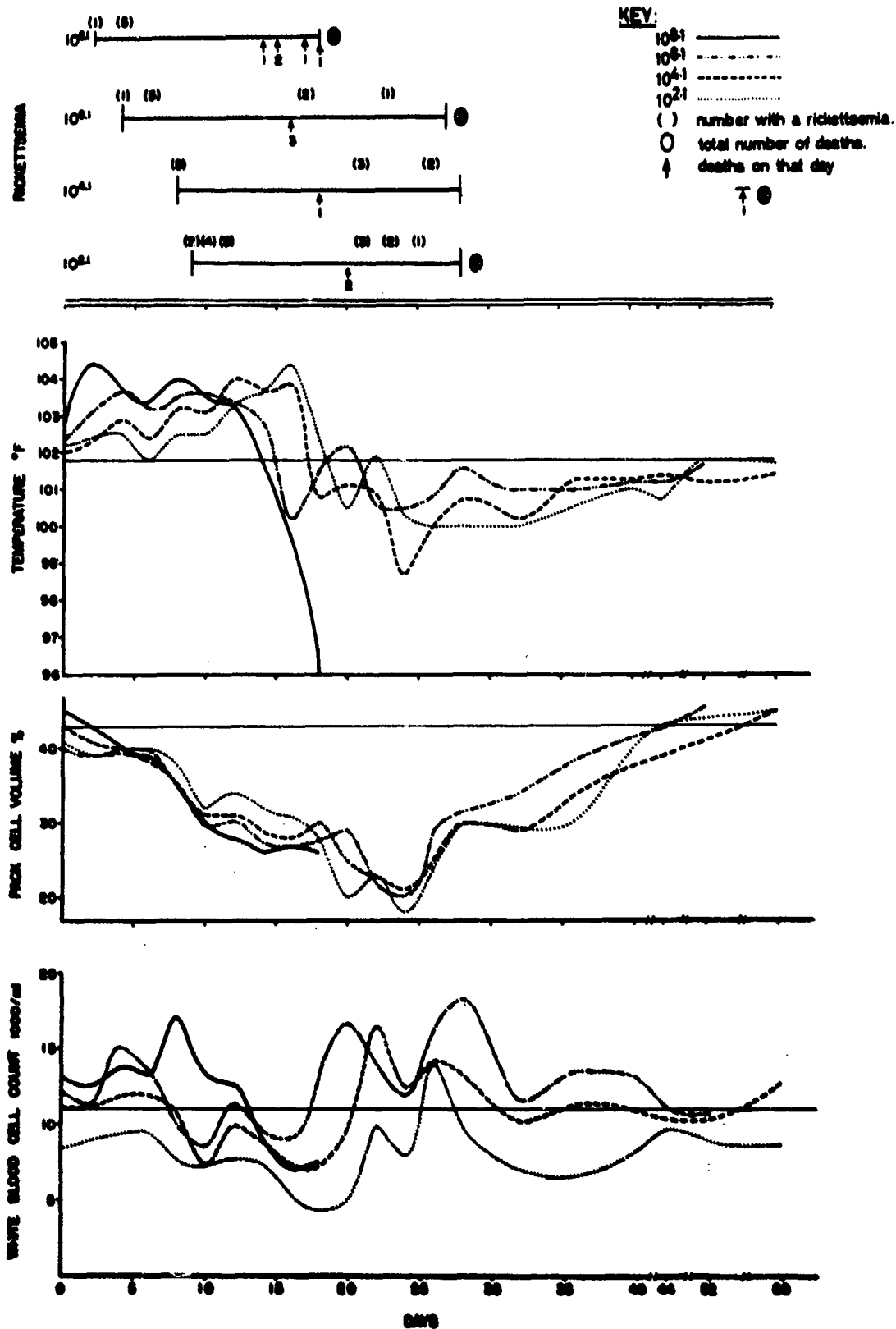if the eschars produced in the silvered leaf-monkeys were identical to

Fig. 5 Effect of Challenge Dose on Scrub Typhus Infection in Silvered
       Leaf-monkeys.

Table 9

Effect of Dose and Strain on Eschars in Silvered Leaf-monkeys; Per Cent of the Animals
Inoculated and Surviving at the Given Time Periods with Eschars

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Strain	Dosage (MYPLD or ID_{50})	Day Post-inoculation														No. Living @ 42 days
		3	4	5	6	7	11	12	14	16	18	20	22	30	42	
Karp	$10^{8.1}$	20	100	100	100	100	100	100	100	100	-*	-	-	-	-	0
	$10^{6.1}$	0	20	100	100	100	100	100	80	25	0	0	0	0	0	2
	$10^{4.1}$	0	0	0	40	80	80	60	60	20	0	0	0	0	0	4
	$10^{2.1}$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Gilliam	$10^{7.5}$	20	100	100	100	100	100	100	100	100	-	-	-	-	-	0
	$10^{5.5}$	0	20	80	100	100	100	100	100	100	100	100	50	0	0	2
	$10^{3.5}$	0	0	0	40	80	80	80	80	80	40	25	25	25	0	4
	$10^{1.5}$	0	0	0	0	0	60	100	100	100	100	100	100	50	50	2

-*: All animals receiving doses of $10^{8.1}$ were dead by day 18.

Note: Inoculation was intradermal (0.1 ml) with Karp inoculated on the inside of one thigh and Gilliam in the other thigh.

those in humans. Ten animals were inoculated with doses of $10^{7.6}$ $MIPLD_{50}$'s of the Karp strain and $10^{8.5}$ $MIPID_{50}$ of the Gilliam strain while another 10 animals received $10^{5.6}$ $MIPLD_{50}$ of Karp and $10^{6.5}$ $MIPID_{50}$'s of Gilliam and 4 served as uninoculated controls. Figure 6 lists the clinical data on this group of animals. All surviving monkeys were euthanized on days 21 and 22 and the four uninoculated controls on day 23. The data are remarkably parallel to that obtained on the 20 animals which received varying doses of Karp and Gilliam (see above and Figure 5). This demonstrates the predictability and reproducibility of scrub typhus infection in the silvered leaf-monkey given a known dose and strain.

This study was done in collaboration with the Department of Experimental Pathology, US Army Medical Component, SEATO. The final details of the histopathology are not available yet. However, some conclusions can be made: Several of the lymph nodes draining the site of inoculation of the Gilliam strain were necrotic on gross examination but none of those draining the site of the Karp strain showed necrosis. The histopathology of the infection of tissues and eschars is remarkably similar to that observed in humans (personal communication Dr. De Paoli, U.S. Army Medical Component, SEATO).

Table 10 gives the eschar data in this group of monkeys. Both strain and dose affected eschar formation and duration. Gilliam eschars lasted for a longer period.

Rechallenge Experiments: The seven animals that survived the dose response experiment (see above) with Karp and Gilliam were followed clinically and serologically for 180 days. They were then inoculated with homologous as well as heterologous strains (see Figure 7 through 14 for clinical pathology data). By 40 days post-challenge, all 7 surviving animals had returned to normal following their first challenge. One animal (#65), Figure 7, had a significant decrease in PCV again between 52 and 142 days but then returned to normal by 161 days. At 101 days post-inoculation, the seven surviving animals were started on tetracycline at the dosage of 125 mg. (16 mg/lb) orally once a day plus 0.5 mg/ml in drinking water for 8 days. This was immediately followed with oral doxycycline 25 mg. (3 mg/lb) for 2 days and then at 15 mg (2 mg/lb) for another 5 days. They remained on tetracycline in the drinking water (0.5 mg/ml) for another 20 days or until 134 days post-challenge at which time all therapy was stopped.

To determine if the surviving animals were cleared of the infection, an inguinal lymph node was excised from both the right and left sides at 160 days post-challenge or 26 days post treatment. Of the 14 inguinal lymph nodes of the 7 monkeys, two from different monkeys (#40, Fig. 11 and #53, Fig. 12) were positive by mouse inoculation and challenge. Antigenic analysis by FA revealed that animal #40 was infected with both Karp and Gilliam while #53 was infected only with Karp. Thus 2 of the seven survivors (#40 and #53) were still chronically infected at 160 days post-inoculation and 26 days following treatment with both tetracycline and doxycline. It is assumed that the remaining 5 animals were not chronically infected.

Fig. 6 Response of Silvered Leaf-monkeys to High Challenge Doses of
R. tsutsugamushi.

Table 10

The Effect of Strain and Dosage on Eschar Formation in a Group of 20
Silvered Leaf-monkeys; Per Cent of the Animals Inoculated and
Surviving at the Given Time Periods with Eschars

Strain	Dosage ($MIPLD$ or $ID_{50's}$)	Day Post-inoculation							
		4	5	6	12	14	16	18	20
Karp	$10^{7.6}$	80*	100	100	100	100	100	100	83
	$10^{5.6}$	10	70	100	100	90	60	50	29
Gilliam	$10^{8.5}$	80	100	100	100	100	100	100	100
	$10^{5.5}$	0	60	100	100	100	90	90	86

Note: Inoculation was intradermal (0.1 ml) with Karp inoculated on
the inside of one thigh and Gilliam in the other thigh.
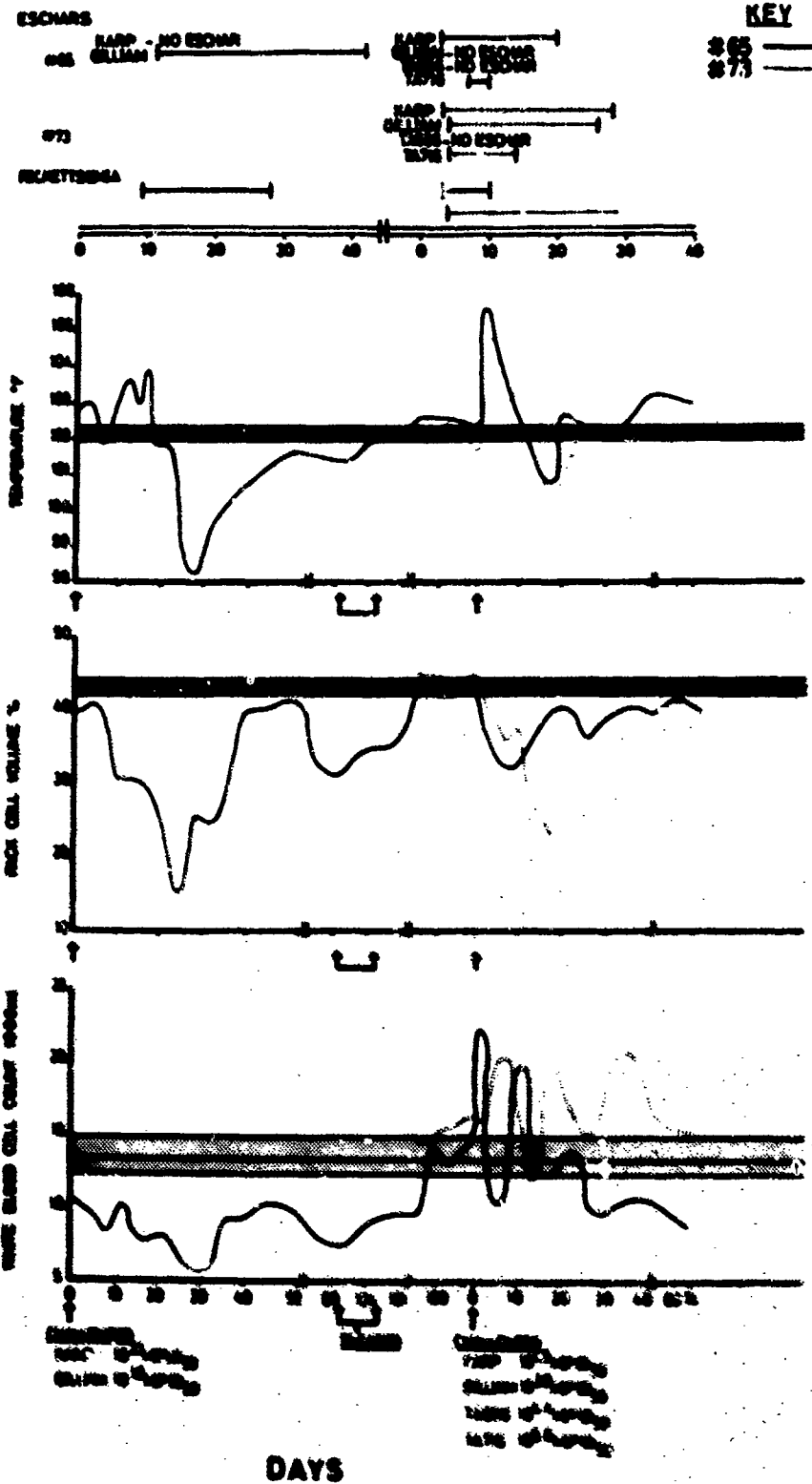
Fig. 7 Effect of Immunity on Scrub Typhus Infection; Homologous-beta against Rechallenge; High Dose.

Fig. 9 Effect of Immunity on Scrub Typhus Infection; Homologous-heterologous
Rechallenge; Medium Dose.

Fig. 9 Effect of Immunity on Scrub Typhus Infection; Homologous-heterologous Rechallenge; Low Dose.
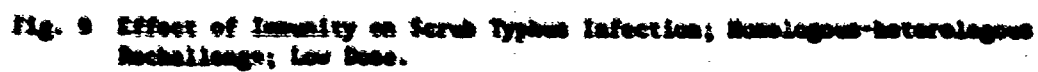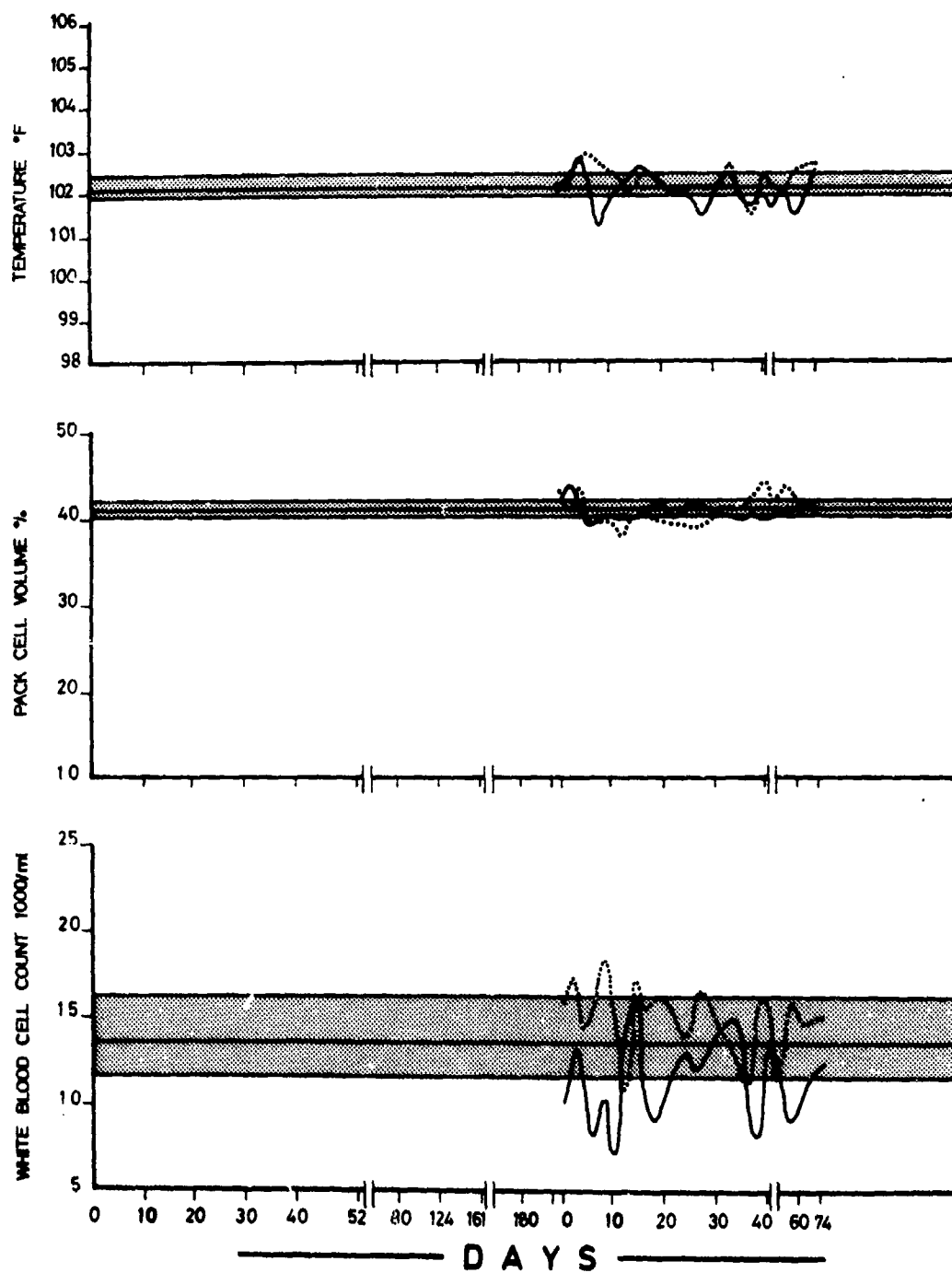
Fig. 10 Effect of Immunity on Scrub Typhus Infection; Uninoculated Controls.
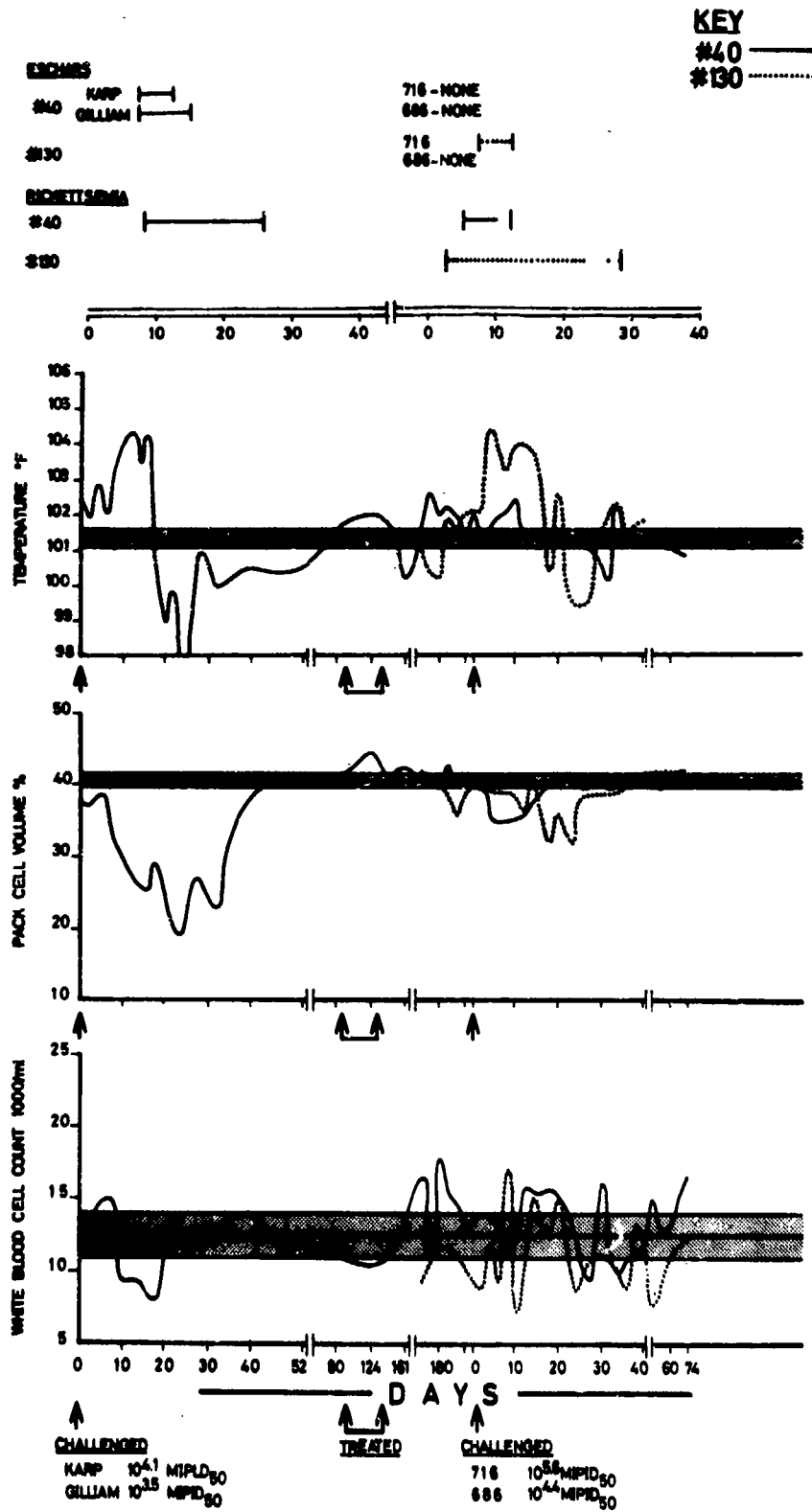
Fig. 11   Effect of Immunity on Scrub Typhus Infection; Heterologous
Rechallenge; High Dose.
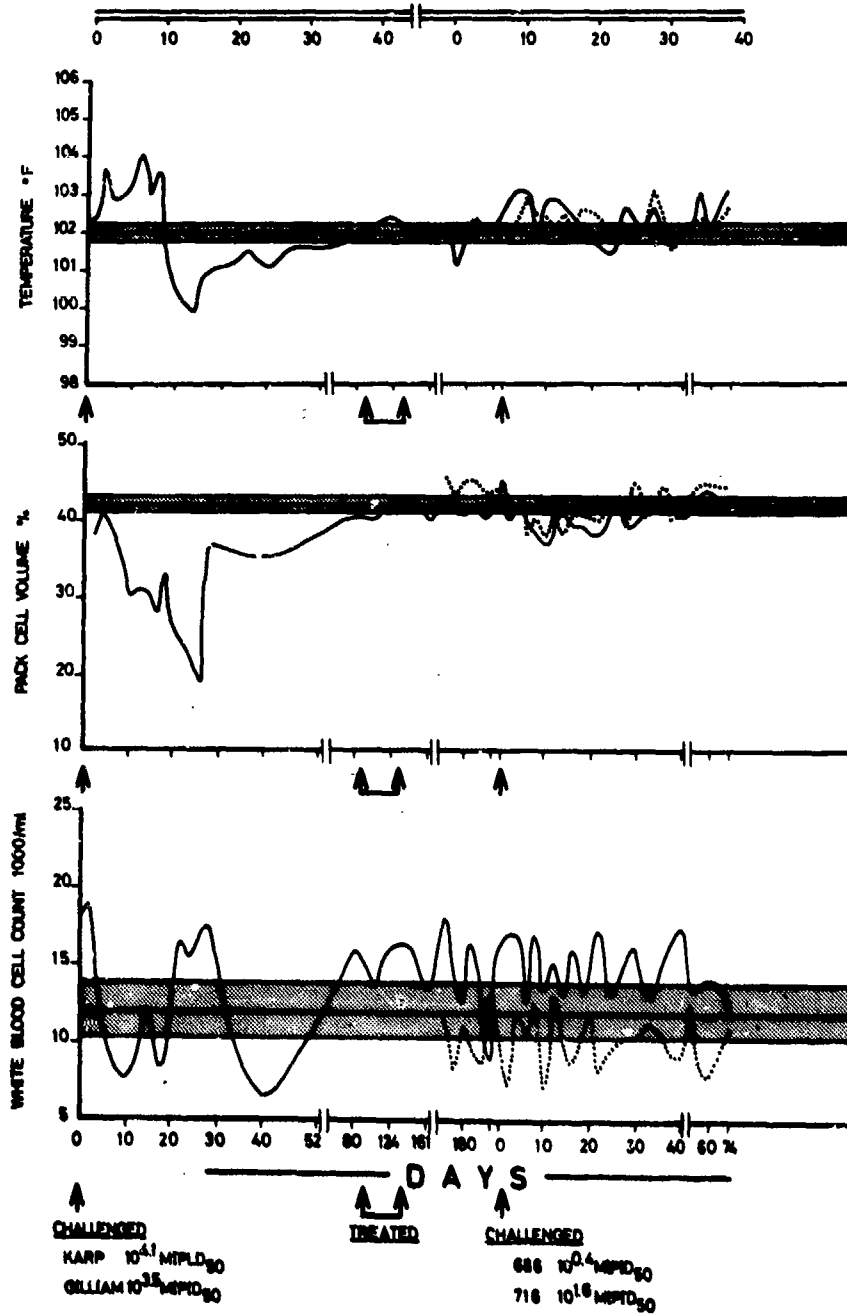
Fig. 12 Effect of Immunity on Scrub Typhus Infection; Heterologous Rechallenge; Medium Dose.

Fig. 13 Effect of Immunity on Scrub Typhus Infection; Heterologous
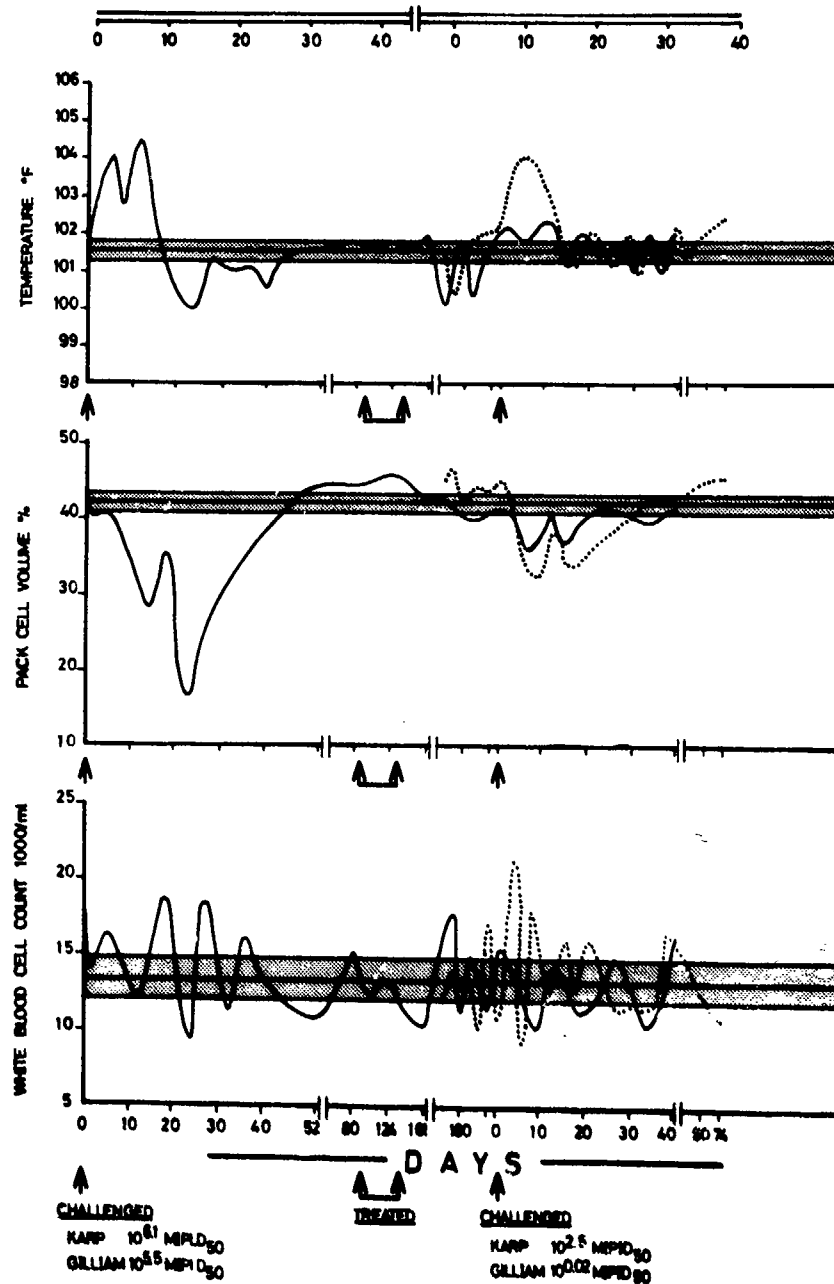   Rechallenge; Low Dose.

Fig. 14 Effect of Immunity on Scrub Typhus Infection; Homologous
Rechallenge; Medium Dose.

At 189 days post-challenge, the seven surviving animals (immunes) were rechallenged. At the same time non-immunes were inoculated with the same strains. See Figures 7 through 14 for challenge strains and dosages. The seven immunes were given rechallenge doses and strains at random and without regard to the original challenge dosage of Karp and Gilliam. The dosages of the Karp and Gilliam strains were much lower than intended because the stock cultures had lost considerable potency; this was unknown to us at the time of challenge.

The data on 3 immune animals (#65, #64, and #47) receiving the homologous-heterologous rechallenge, their 3 nonimmune controls (#73, #109 and #76) and the uninoculated controls (#71 and #128) are presented in Figures 7 through 9. In all cases the responses (rickettsemia, temperature, PCV and WBC) was of a much lesser degree in the three immunes than in their respective inoculated control. Although all three immune animals (#65, #64 and #47) ran a rickettsemia it was of a shorter duration than in controls. None of the three immune animals demonstrated listlessness, anorexia, or weakness. In one case the immune animal (#65) failed to develop a Gilliam eschar while its inoculated control (#73) did. The duration of the TA 716 eschar was shorter in #65 than in the nonimmune, #73. Isolates were obtained from day 4 blood specimens on #65 and #73. Karp was isolated from the immune animal, #65, while Karp and TA 716 were isolated from the nonimmune control, #73. The clinical data and rickettsemia data indicate that immune monkey #65 had a very low order of protection against the Karp strain. Indeed #65 only had a titer of 1:10 against the Karp strain at the time of rechallenge.

The clinical data on 3 immune animals (#40, #53 and #36) receiving the heterologous rechallenge, their 3 nonimmune inoculated controls (#130, #114 and #116) and the 1 receiving the homologous rechallenge (#68) and its nonimmune inoculated control (#124) are presented in Figures 11 through 14. The responses were dose dependent. The responses (temperature and PCV) were much less in 2 immunes (#40 and #68) than in their nonimmune controls, Figures 11 and 14. In the two other sets of animals (#53 - #114 and #36 - #116) the degree of illness and response was so mild in even the nonimmune inoculated controls that no differences were observed, Figures 12 and 13. In 3 cases the duration of the rickettsemia was shorter in the immunes than in their respective controls and in one case the immune animal (#36, Fig.13) failed to develop any rickettsemia. Only the one nonimmune animal (#130) receiving the highest challenge dose of the heterologous strains (TA 686 and TA 716) developed a TA 716 eschar; the immune animal receiving the same dose (#40) failed to develop an eschar. In contrast to the case of the immune animal (#65) in Figure 7, the immune animal (#68, Fig.14) receiving the homologous rechallenge with Karp and Gilliam failed to develop a Karp eschar while the nonimmune inoculated control (#124) developed a Karp eschar that lasted for approximately 7 days. It should be pointed out the #68 received a dose of $10^{2.5}$ MIPID$_{50}$ of Karp. In addition #68 had titer to Karp of 1:40 at the time of rechallenge in contrast to #65 whose titer was only 1:10 (see below).

The serological data to all nine prototype strains of *R. tsutsugamushi* for the 3 immune (#65, #64, and #47) and their respective nonimmune inoculated controls (#73, #109 and #76) receiving the homologous-heterologous challenge are presented in Figures 15 through 17. For the immune animals both their primary response to the initial challenge (Karp and Gilliam) and the secondary serological response following rechallenge with homologous (Karp, Gilliam)-heterologous (TA 686 and TA 716) strains at three doses are given. The immune animals, #65 and #64, which received the high and medium doses responded rapidly to both homologous and heterologous antigens. The immune animal, #47, that received the low dose failed to respond serologically (Fig.17), while its nonimmune inoculated control, #76, responded.

The serological data to all nine prototype strains of *R. tsutsugamushi* for 3 immunes (#40, #53, and #36) and their nonimmune inoculated controls (#130, #114 and #116), which received a heterologous challenge with TA 686 and TA 716, are presented in Figures 18 through 20. Again, at the high and medium doses, the immune animals responded rapidly; within 10 days. The two animals, #36 and #116, that received the low dose failed to respond, see Figure 20. This is interpreted to mean that the dosage of TA 686 and TA 716 was too low to cause an infection even in the nonimmune animal, #116.

The serological data, Figure 21, on the two animals receiving the homologous rechallenge (Karp and Gilliam) agrees completely with the clinical data, see Figure 14. The immune animal, #68, was completely protected against rechallenge with homologous strains.

In conclusion, by combining strains, significant titers and protection were obtained to the minor components as well as the major antigenic components of the strains. Complete clinical protection was demonstrated at 6 months to homologous, homologous-heterologous, and heterologous challenge which extended in three cases to the prevention of eschar formation. In the immune animals that did develop changes in body temperature, WBC's or PCV, the degree of change was much less and the duration was much shorter than that in nonimmune controls.

Candidate Vaccine Strains in Silvered Leaf-monkeys: The work is not complete yet, but a group of 21 silvered leaf-monkeys was infected with the four candidate vaccine strains, TA 763, TH 1817, TC 586 and TA 678 at a high (10^6), medium (10^4) and low (10^2) dose. Four separate sites of inoculation (ID) were used. The data available are reported in Table 11 and Figure 22. Two strains, TC 586 and TA 678, failed to produce eschars even at a high dose (TC 586 = $10^{6.0}$ MIPID$_{50}$'s and TA 678 = $10^{5.5}$ MIPID$_{50}$'s) while the other two produced eschars only at the high dose (TA 763 - $10^{6.2}$ MIPID$_{50}$'s and TH 1817 = $10^{5.0}$ MIPID$_{50}$'s). When the data are plotted for the relationship between the day that rickettsemia began and the dosage a straight line fit was obtained (Figure 22).
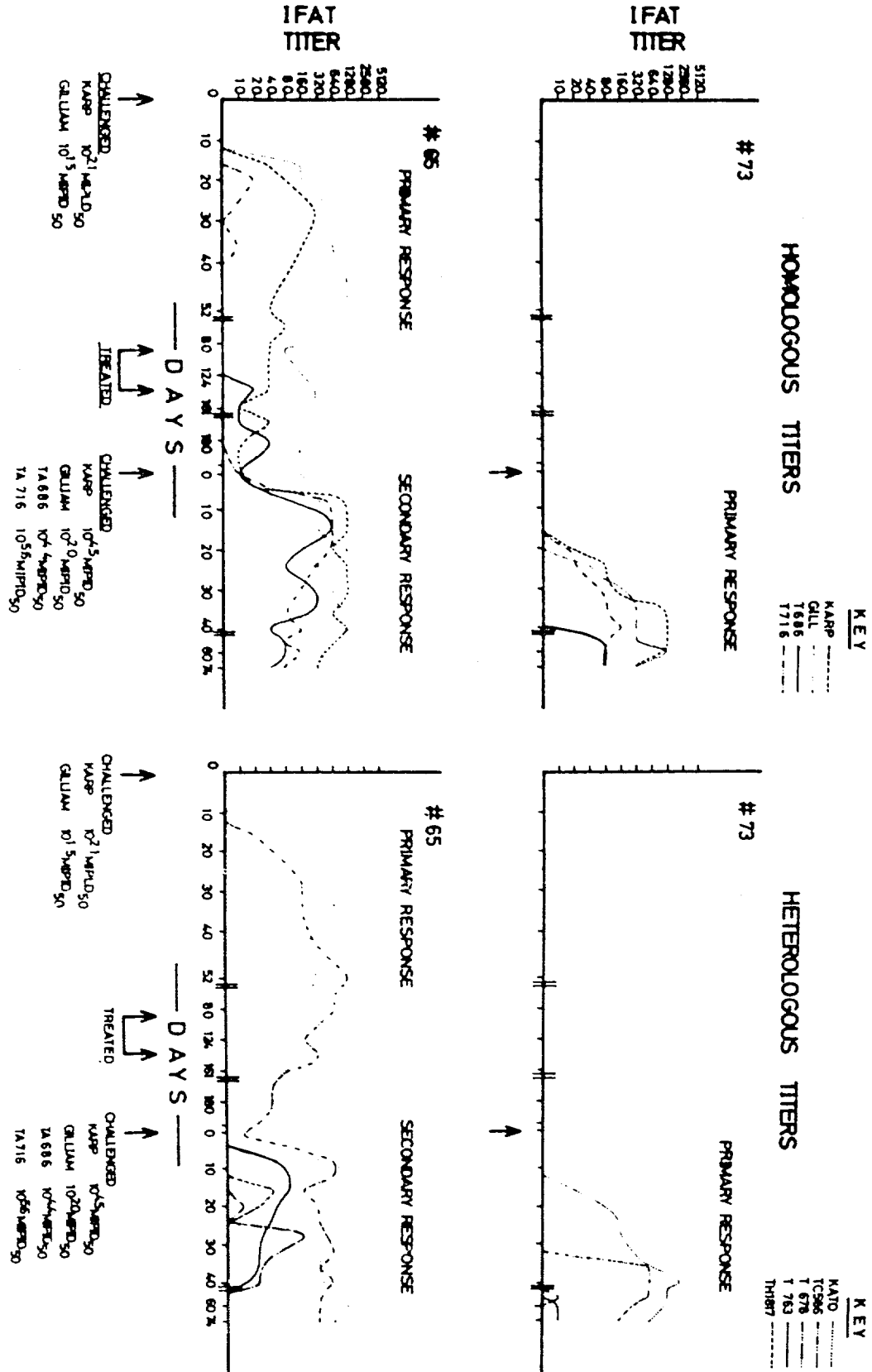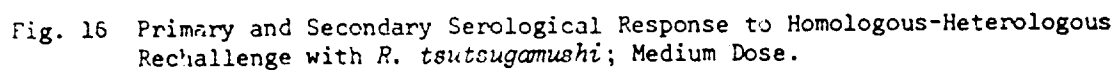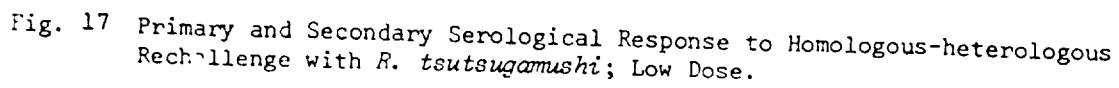
Fig. 15 Primary and Secondary Serological Response to Homologous-heterologous
Rechallenge with *R. tsutsugamushi*; High Dose.

Fig. 16 Primary and Secondary Serological Response to Homologous-Heterologous
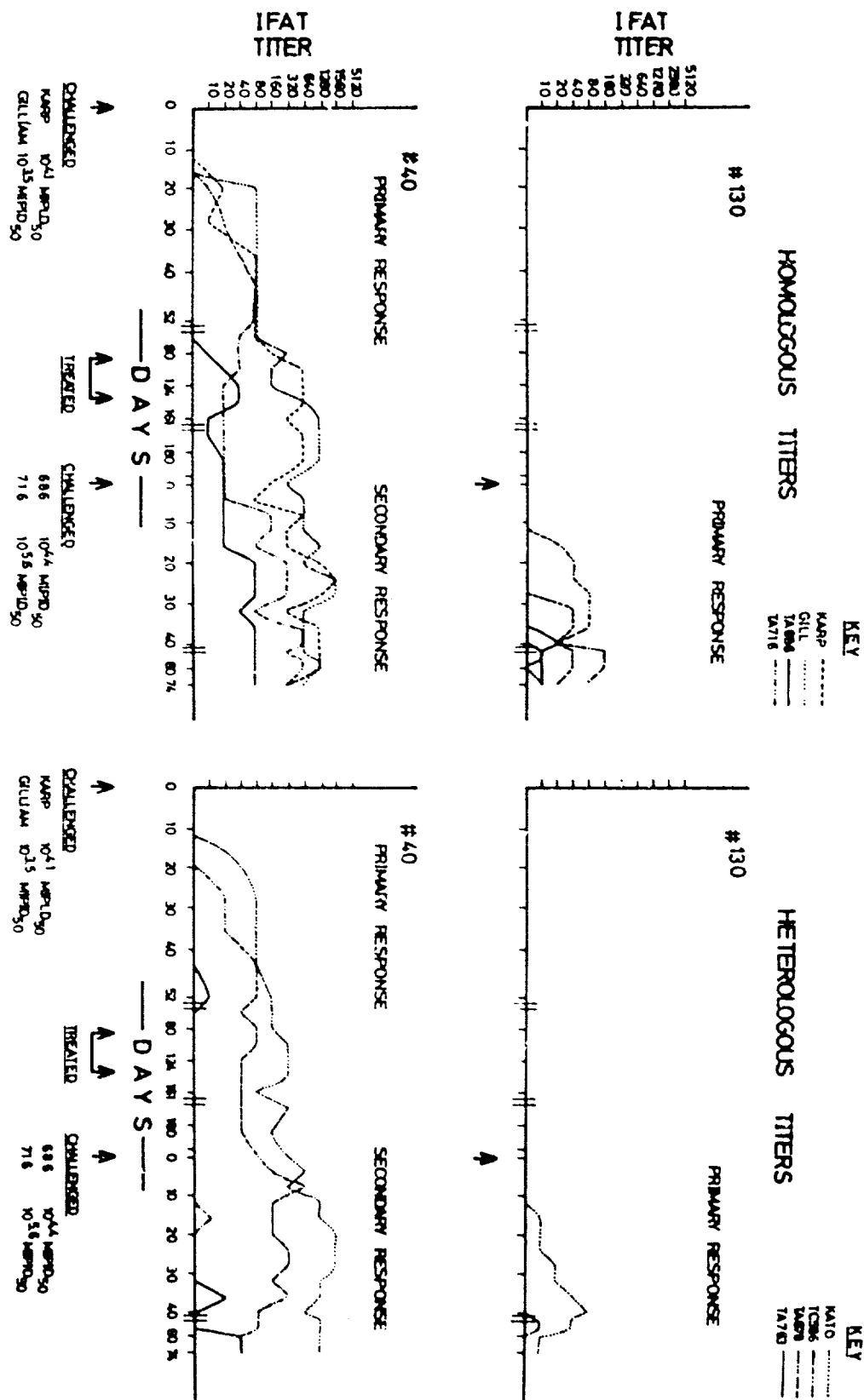   Rechallenge with *R. tsutsugamushi*; Medium Dose.

Fig. 17 Primary and Secondary Serological Response to Homologous-heterologous Rechallenge with *R. tsutsugamushi*; Low Dose.

Fig. 18 Primary and Secondary Serological Response to a Heterologous
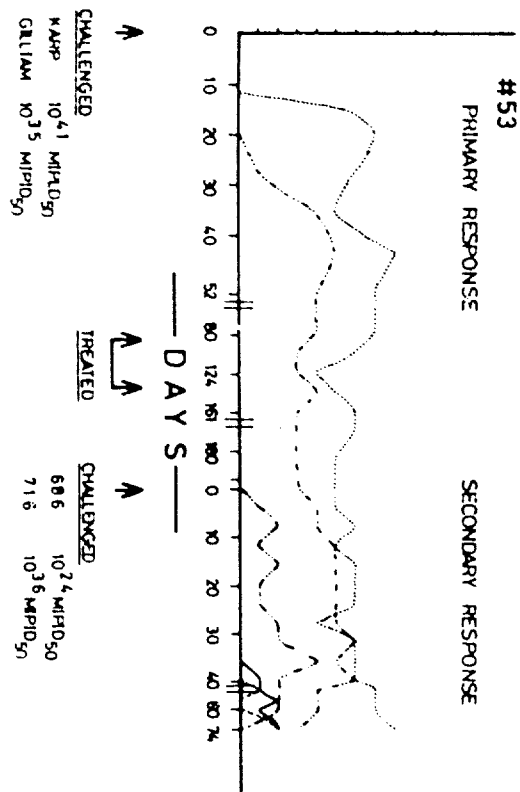   Rechallenge with *R. tuetsugamushi*; High Dose.

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Fig. 19 Primary and Secondary Serological Response to a Heterologous Rechallenge with *R. tsutsugamushi*; Medium Dose.

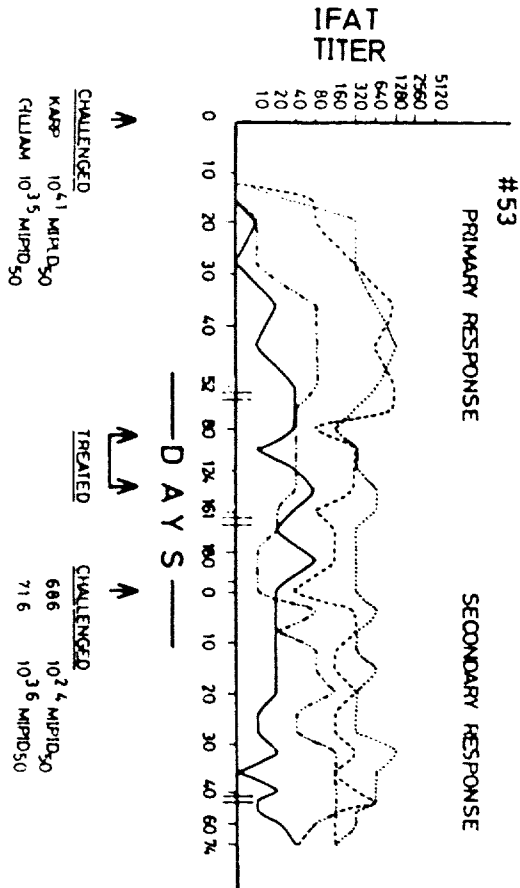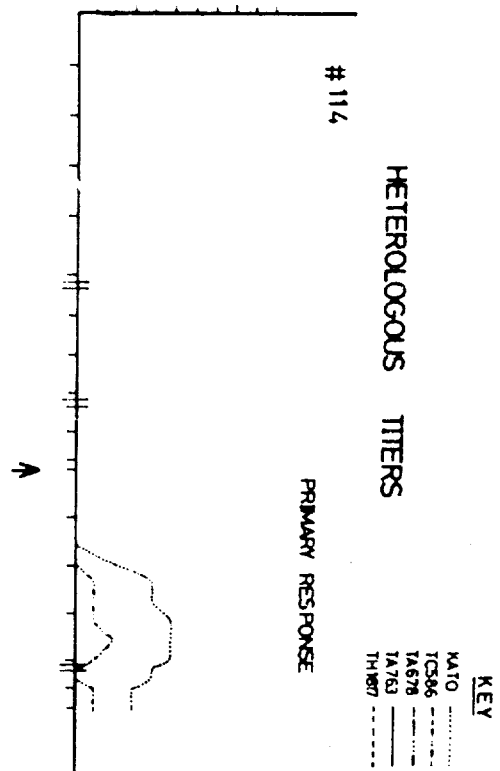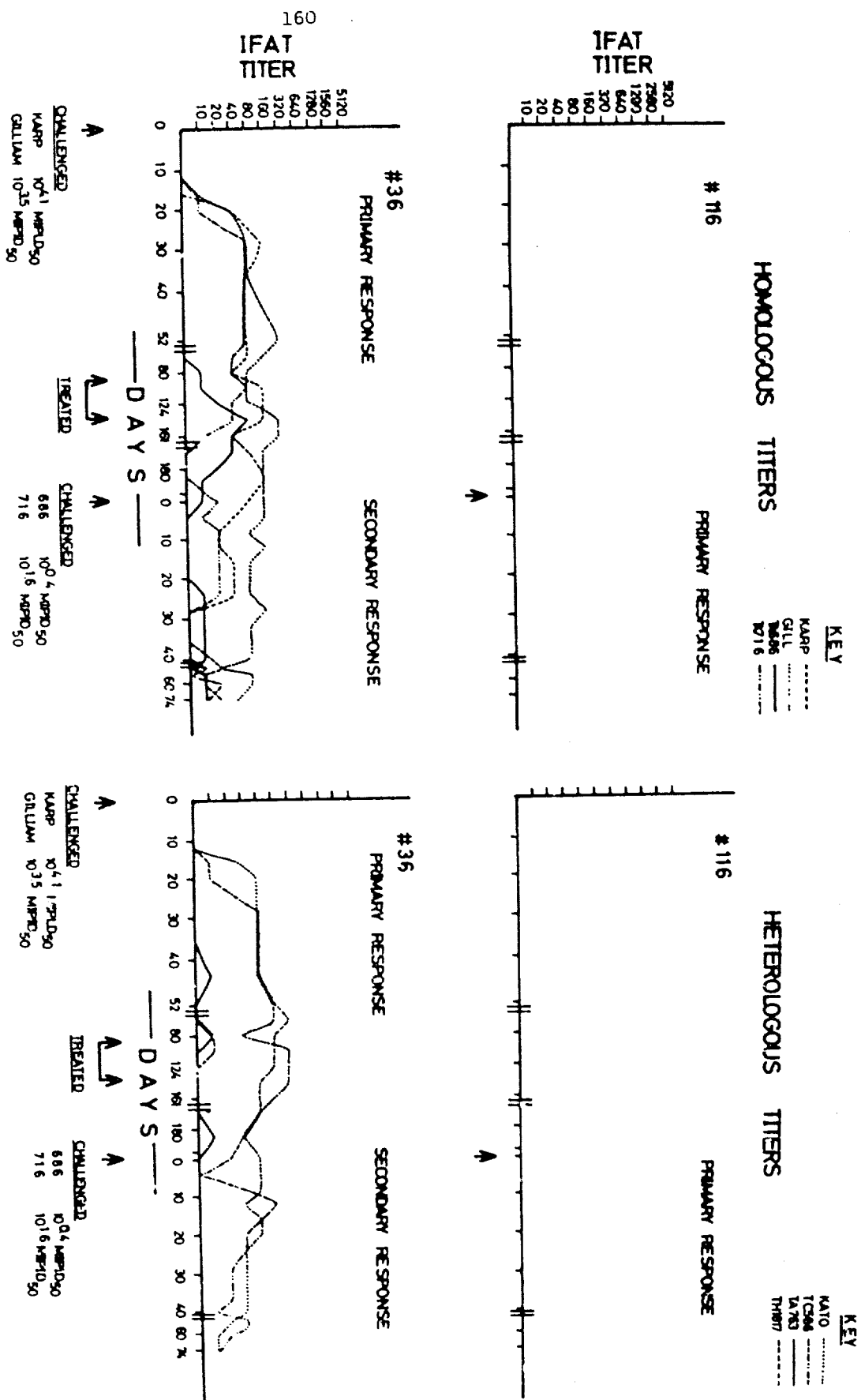Fig. 20 Primary and Secondary Serological Response to 2 Heterologous Rechallenge with *R. tsutsugamushi*; Low Dose.
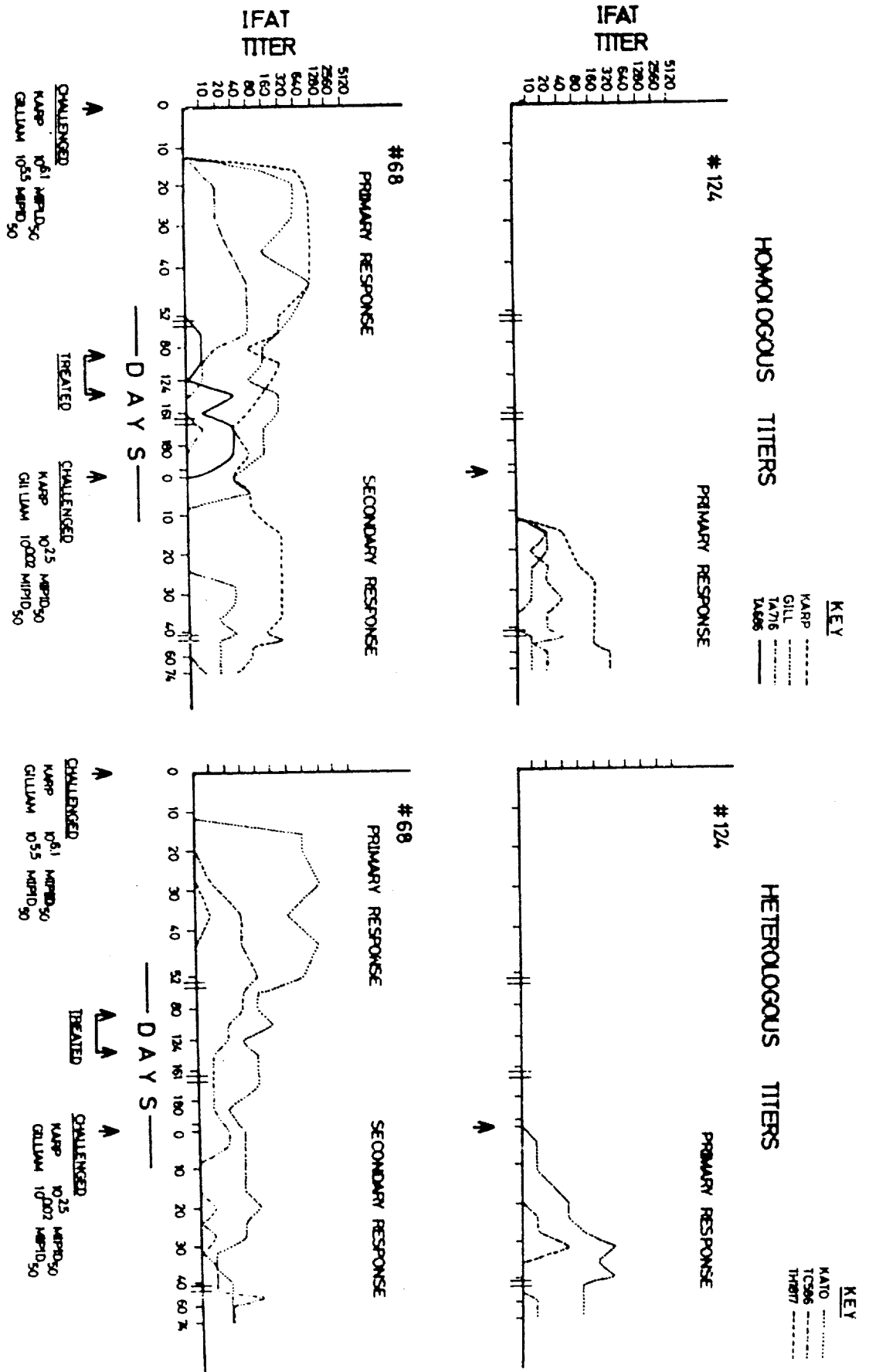
Fig. 21 Primary and Secondary Serological Response to a Homologous Rechallenge with *R. tsutsugamushi*; Medium Dose.

EFFECT OF CHALLENGE DOSE OF FOUR CANDIDATE VACCINE STRAINS ON THE ONSET OF RICKETTSEMIA
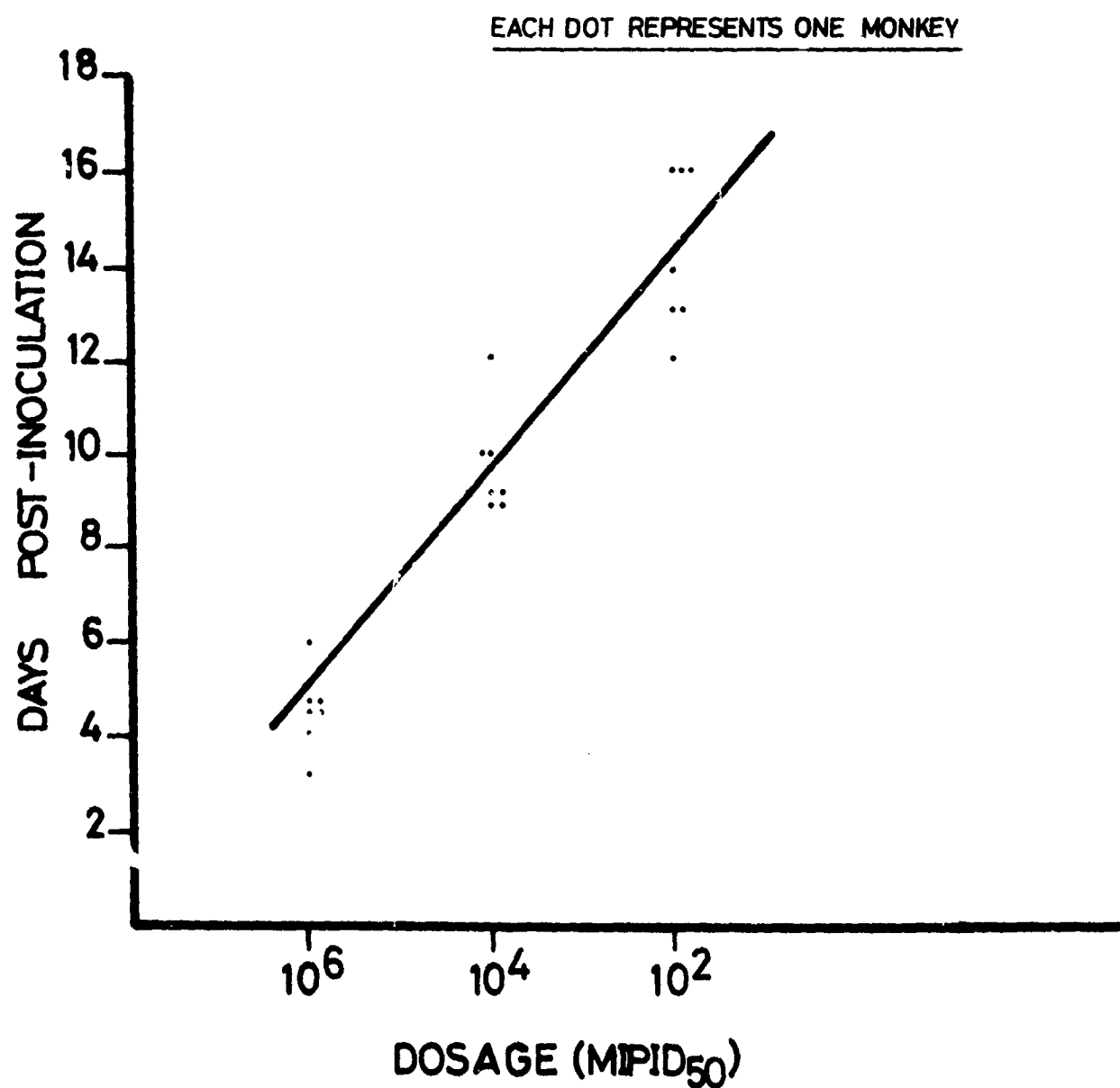
Figure 22

Table 11

Effect of Dosage and Strain on Eschar Formation

Dosage (MIPLD or ID_{50}'s)	Eschar Formation			
	Strain			
	TA 763	TH 1817	TC 586	TA 678
10^6	7/7[1]	4/7	0/7	0/7
10^4	0/7	0/7	0/7	0/7
10^2	0/7	0/7	0/7	0/7

1. Number developing eschars over total number.

None of the animals became as sick as from the Gilliam strain at comparable doses, nor did any of the 21 animals die before 30 days. Also, the degree of hyperthermia and anemia was much less. These 21 animals will be treated with doxycycline for three weeks beginning at 110 days and then rechallenged with heterologous strains at high doses at 180 days post-inoculation.

Summary of Eschar Formation in Silvered Leaf-monkeys: A summary of the effect of strain and dosage on eschar formation for all silvered leaf-monkeys challenged is given in Table 12. The effect of immunity on eschar formation was shown in Figure 23. Three strains, TA 678, TA 686 and TC 586 failed to produce eschars at any of the dosages while two others, TA 716 and TA 763 produced eschars at dosages of 10^6 and not below. Gilliam produced eschars in doses as low 10^2 $MIPID_{50}$'s and Karp did also in 2 out of 7 cases. Thus, eschar formation is strain and dose dependent in silvered leaf-monkeys. This may explain the discrepancies that exist in the literature over the percentage of eschars observed in different areas in human cases of scrub typhus.

The effect of immunity on eschar formation and duration is illustrated in Figure 23. Not only did not the immune animal not develop a Gilliam eschar but the duration of the Karp eschar was shortened. The challenge dosages were Karp $10^{4.5}$, Gilliam $10^{2.0}$, TA 716 $10^{5.6}$ and TA 686 $10^{4.4}$ $MIPID_{50}$'s (the latter two not shown in Figure 12). TA 686 produced only a low order of inflammation at the site of injection even in the nonimmune animal. Note that by day 24 the Karp eschar was completely healed in the immune animal while in the nonimmune animal, healing had not started.

The Response of Gibbons to Scrub Typhus Infection: Two gibbons were challenged during this reporting period, one was a nonimmune animal which was challenged ID with $10^{4.3}$ $MIPID_{50}$ of the Karp strain and one was an immune animal rechallenged with heterologous strains. The data on the immune animal was reported last year, but are repeated in Figure 24 for comparison. The immune animal which had received a Karp challenge ($10^{7.5}$ $MIPLD_{50}$'s) was completely protected when rechallenged with the heterologous Kato and Gilliam strains at 60 days as determined by clinical course. However, the animal did develop eschars, and ran a shorter and intermittent rickettsemia. Because the Kato and Gilliam strains were injected together in the same site, it is impossible to determine with certainty which one caused the eschars.

The animal that received Karp, ID, at $10^{4.3}$ $MIPLD_{50}$ became very sick and we think would have died if not treated with chloramphenicol. As can be seen in Figure 24 the temperature immediately dropped and the animal made a complete recovery within 48 hours. Interestingly enough, chloramphenicol did not clear the rickettsemia which continued for 11 days. One isolate during this 11 day period was proven by back-challenge to be *R. tsutsugamushi*. By day 60 this animal had completely recovered and the PCV, WBC and temperature were all back to normal.

Table 12

Effect of Strain and Dose on Eschar Formation in 94 Nonimmune Silvered Leaf-monkeys

Dosage MIPLD or ID$_{50}$'s ($\pm$ 10$^{0.5}$)	Karp	Gilliam	Kato	Prototype Strains					
				TA 678	TA 686	TA 716	TA 763	TH 1817	TC 586
10^8	15/15[1]	15/15	-	-	-	-	-	-	-
10^6	15/15	15/15	-	0/7	-	2/2	7/7	4/7	0/7
10^4	10/11	4/5	1/1	0/7	0/2	0/2	0/7	0/7	0/7
10^2	2/7	6/6	-	0/7	0/2	0/2	0/7	0/7	0/7
Total	42/48	40/41	1/1	0/21	0/4	2/6	7/21	4/21	0/21

1. Number developing eschars/total number inoculated intradermally with 0.1 ml.

IMMUNE
(#65)

NON-IMMUNE
(#73)
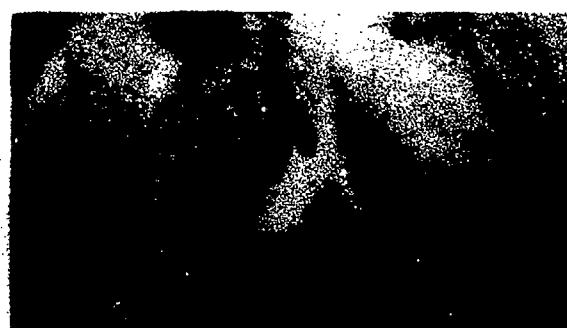
DAY- 4
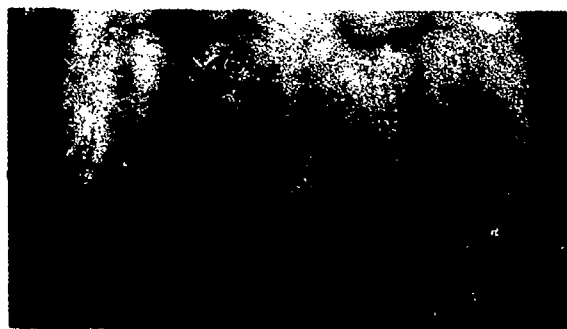
DAY-16
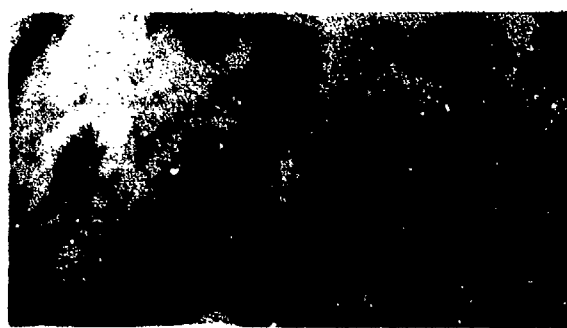
DAY-24

Fig.23 Karp & Gilliam Eschars in a Immune & Nonimmune Silvered Leaf Monkey.

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Fig. 24 The Response of Nonimmune & Immune Gibbons to an Interadermal
Challenge with *R. tsutsugamushi*.

Summary of Primate Challenge with all Nine Prototype Strains

1.   The silvered leaf-monkey is susceptible to *R. tsutsugamushi* and appears to be an excellent model for human scrub typhus.

2.   The various responses (anorexia, listnessless, weakness, temperature, WBC, PCV, eschar formation, time of occurrence of rickettsemia, and death) were all remarkably uniform with given strains. All were dose dependent.

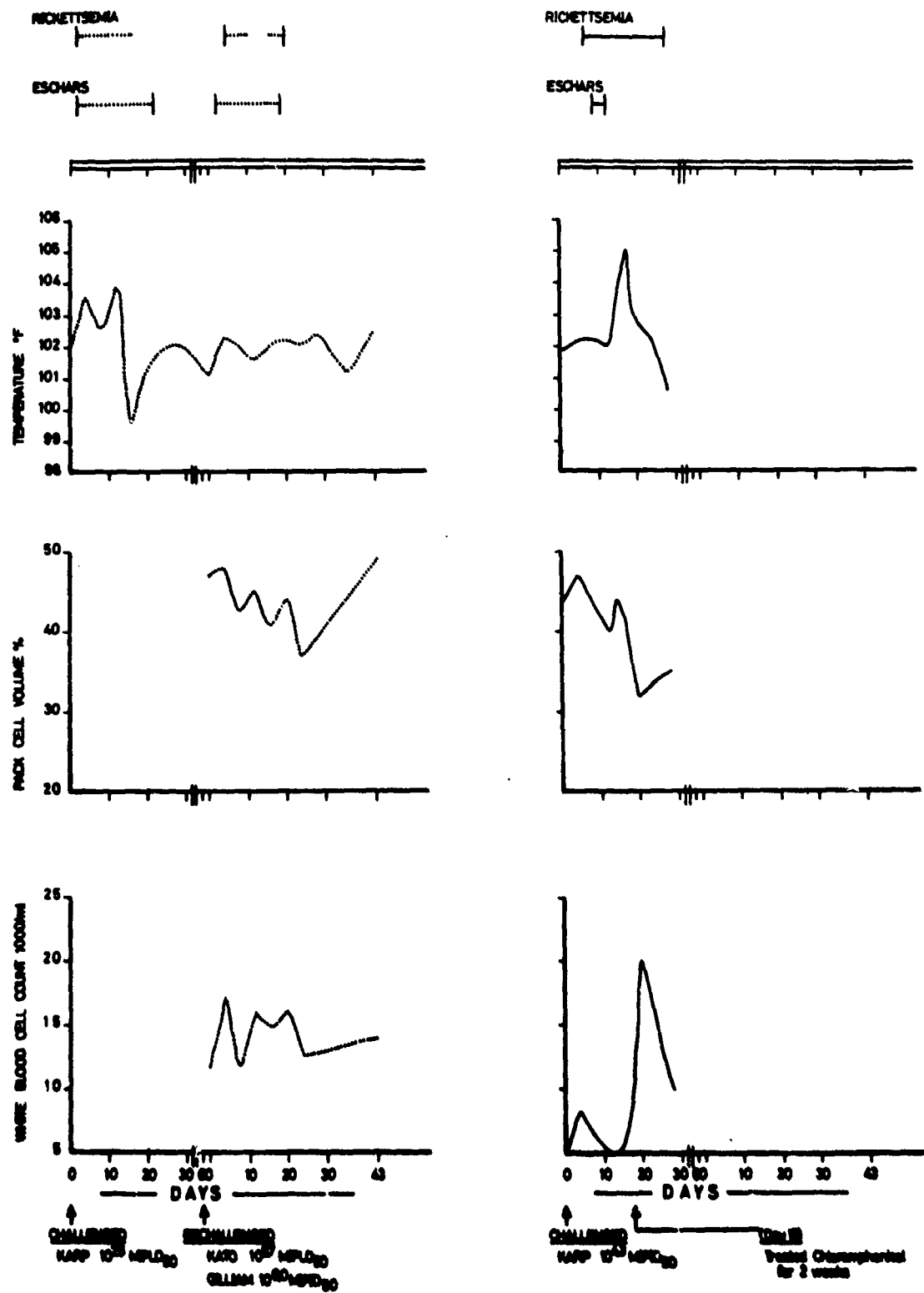3.   Clinical illness, death, eschar formation and duration are all strain dependent. Three strains did not produce any eschars.

4.   Significant titers were obtained to the minor as well as major antigenic components in seven animals surviving a Karp and Gilliam challenge. In the majority the animals there was significant levels of antibody to six of the nine prototype strains at 180 days post-challenge.

5.   Complete protection from clinical illness was obtained in seven immune animals challenged 189 days post-inoculation with homologous, homologous-heterologous and heterologous combinations of the prototype strains. Eschar formation and duration was also effected by the immune status of the animals.

6.   The serologic response to rechallenge was very rapid in the animals receiving the homologous-heterologous rechallenge and by eight days the majority of the animals given high doses had significant levels of antibody to all nine prototype strains even when they had only received four of the nine. In the nonimmunes, the response to the homologous strains was 20+ days and to the heterologous 40+ in most cases.

7.   Gibbons are also susceptible to infection with *R. tsutsugamushi* and develop eschars with certain strains. The one animal rechallenged with heterologous strains at 60 days was resistant to the rechallenge.

Antigenic Stability:

In Silvered Leaf-monkeys: From 12 of the previous animals isolates were made at 60 days post-inoculation. These isolates were checked to determine whether or not the strains had remained antigenically stable (Table 13). In all cases but 1, only those strains inoculated were recovered. The exception is an animal that received only Kato, in which Karp was recovered in addition to Kato. The procedure was repeated twice using two different sets of mice and cell preparations (peritoneal smears as well as glycogen prepared cells) with separately prepared conjugates and the same results were obtained both times. In neither test was there any cross reaction in the controls.

Table 13

Antigenic Stability of *R. tsutsugamushi* in Silvered Leaf-monkeys as
Determined by Strain Specific FA Conjugates

Challenge Strains	No. of Animals	Strains Isolated at 60 days							
		Karp	Gilliam	Kato	TA 678	TA 763	TA 686	TA 716	TH 1817
Karp	1	+	–	–	–	–	–	–	–
Gilliam	1	–	+	–	–	–	–	–	–
Kato	1	+	–	+	–	–	–	–	–
Karp + Kato	2	+	–	+	–	–	–	–	–
Karp + Gilliam	2	+	+	–	–	–	–	–	–
Kato + Gilliam	1	–	+	+	–	–	–	–	–
Karp + Gilliam + Kato	4	+	+	+	–	–	–	–	–

In the Vector Mite L. (L.) fletcheri: Preliminary results were reported in last year's annual report with three strain specific conjugates to the Karp, Gilliam and Kato strains. The results showed that the positive colony of *L. (L.) fletcheri* contains the Kato strain and may contain Karp also. During the last year these studies were expanded and strain specific conjugates to all nine prototype strains were made available by the Department of Rickettsial Diseases, WRAIR with whom this project was done in collaboration. For details on how the conjugates were prepared, their specificity and the technique see the WRAIR Annual Report 1972. Antisera to the isolates from all the individuals tested by IF were prepared in guinea pigs and sent to WRAIR for strain specific CF tests, see WRAIR Annual Report 1972 for results.

The results obtained with the strain specific conjugates for three lines of the positive *L. (L.) fletcheri* colony are shown in Figure 25. The original pool contained six of the prototype strains. From this pool of 100 chiggers, one adult female was mated and 12 F-1 larvae were fed individually, of these 12, two were reared to adults and served as the progenitors of the colony. One of the F-1 chiggers was infected with Karp and Kato and from the other one only Karp was isolated. From these two individuals, two lines were formed that compose the current colony. The following strain specific conjugates were used to test material from the colony, Karp, Gilliam, Kato, TA 763, TA 678 and TH 1617. The TA 763 conjugate also detects TA 686 and TA 716 while the Gilliam conjugate detects TC 586. Thus, all nine prototype strains other than Kato and Karp were checked for. From F-1 on no strains other than Kato and Karp were detected, although they were tested against all the isolates.

In a Wild Rodent (Rattus annandalei): Since wild rodents may be part of the disease cycle in nature and are frequently infected with *R. tsutsugamushi* it was felt to be necessary to determine the antigenic stability in them. A wild rat, *R. annandalei*, was chosen for this study because they are infected frequently in nature (21% positive blood and 33% positive kidney isolation with 68% positive on IFAT serology) see USAMRU, Malaysia, Annual Report 1971. They were colonized, see Laboratory Animal Section, USAMRU Annual Reports 1971 and 1972, and F-1 and F-2 offspring born in the laboratory were utilized for these studies.

Eight rats were infected with known strains, Karp, 10^4 MIPLD$_{50}$'s and Gilliam, 10^4 MIPID$_{50}$'s, IP. At 3 months, their spleens were removed, and a 20% suspension inoculated into another group of rats. It was also inoculated into mice for peritoneal smears and cells to be used in FA strain analysis and into guinea pigs for sera to be used for CF strain analysis (sent to WRAIR). This was repeated at 6 months post-inoculation for the right kidney and at 9 months for the left kidney at which time the animals were euthanized. The same procedures were carried out on the 2nd and 3rd passage animals, see Table 14. IFAT titers were determined on all animals at 8, 14, 21, 30, 60, 90, 120, 150, 180, 210, 240 & 280 days to

Table 14

Chronic Infection and Antigenic Stability in *Rattus annandalei*

TIME POST-CHALLENGE

Group No.	Rat No.	Passage Levels	Infected with	1 Months				6 Months				9 Months			
				Organ Sampled	Strains Isolated	Titer Karp	Gilliam	Organ	Strains Isolated	Titer Karp	Gilliam	Organ	Strains Isolated	Karp	Titer Gilliam
1	1	1	yolk sac	spleen	Karp - Gilliam	640	160	Right kidney	Karp > Gilliam	2560	40	Left kidney	Karp	640	40
	2	2	spleen of R1	spleen	Karp	640	40	Right kidney	Karp	640	Neg				
	3	3	spleen of R2	spleen	Neg	Neg	Neg								
	4	2	right kidney of R1	spleen	Karp	160	40								
2	5	1	yolk sac	spleen	Karp - Gilliam	160	160	Right kidney	Karp > Gilliam	160	40	Left kidney	Karp	160	40
	6	2	spleen of R5	spleen	Neg	Neg	Neg								
	7**	2	right kidney of R5	spleen	Karp	640	40	(died at 3 months)							
3	8	1	yolk sac	spleen	Gilliam	160	160	(died at 3 months)							
	9	2	spleen of R8	spleen	Neg	Neg	Neg								
4	10	1	yolk sac	spleen	Karp - Gilliam	160	160	Right kidney	Neg	640	160	Left kidney	Karp	160	40
	11	2	spleen of R10	spleen	Karp	640	40	Right kidney	Karp	160	Neg				
	12	2	right kidney of R10	spleen	Neg	Neg	Neg								
	13	3	spleen of R11	spleen	Neg	Neg	Neg								
5	14	1	yolk sac	spleen	Karp - Gilliam	640	160	Right kidney	Karp	2560	160	Left kidney	Karp	160	160
	15	2	spleen of R14	spleen	Neg	Neg	Neg								
	16	2	right kidney of R14	spleen	Neg	160	40								
6	17	1	yolk sac	spleen	Karp - Gilliam	640	40	(died at 3 months)							
	18	2	spleen of R17	spleen	Karp	640	40	Right kidney	Neg	160	Neg				
	19	3	spleen of R18	spleen	Neg	Neg	Neg								
7	20	1	yolk sac	spleen	Karp > Gilliam	160	40	Right kidney	Neg	Neg	Neg	Left kidney	Neg	Neg	Neg
	21	2	spleen of R20	spleen	Neg	Neg	Neg								
	22	2	right kidney of R20	spleen	Neg	Neg	Neg								
8	23	1	yolk sac	spleen	Karp - Gilliam	Neg*	Neg	Right kidney	Neg	Neg	Neg	Left kidney	Neg	Neg	Neg
	24	2	spleen of R23	spleen	Neg	Neg	Neg								
	25	2	right kidney of R23	spleen	Neg	Neg	Neg								

Neg* = Note R23 was infected as evidenced by titers 1:40 to Karp and 1:160 to Gilliam @ 30 and 60 days post-challenge.

7** = Animal died following removal of the spleen and the right kidney was taken and yielded Karp also.
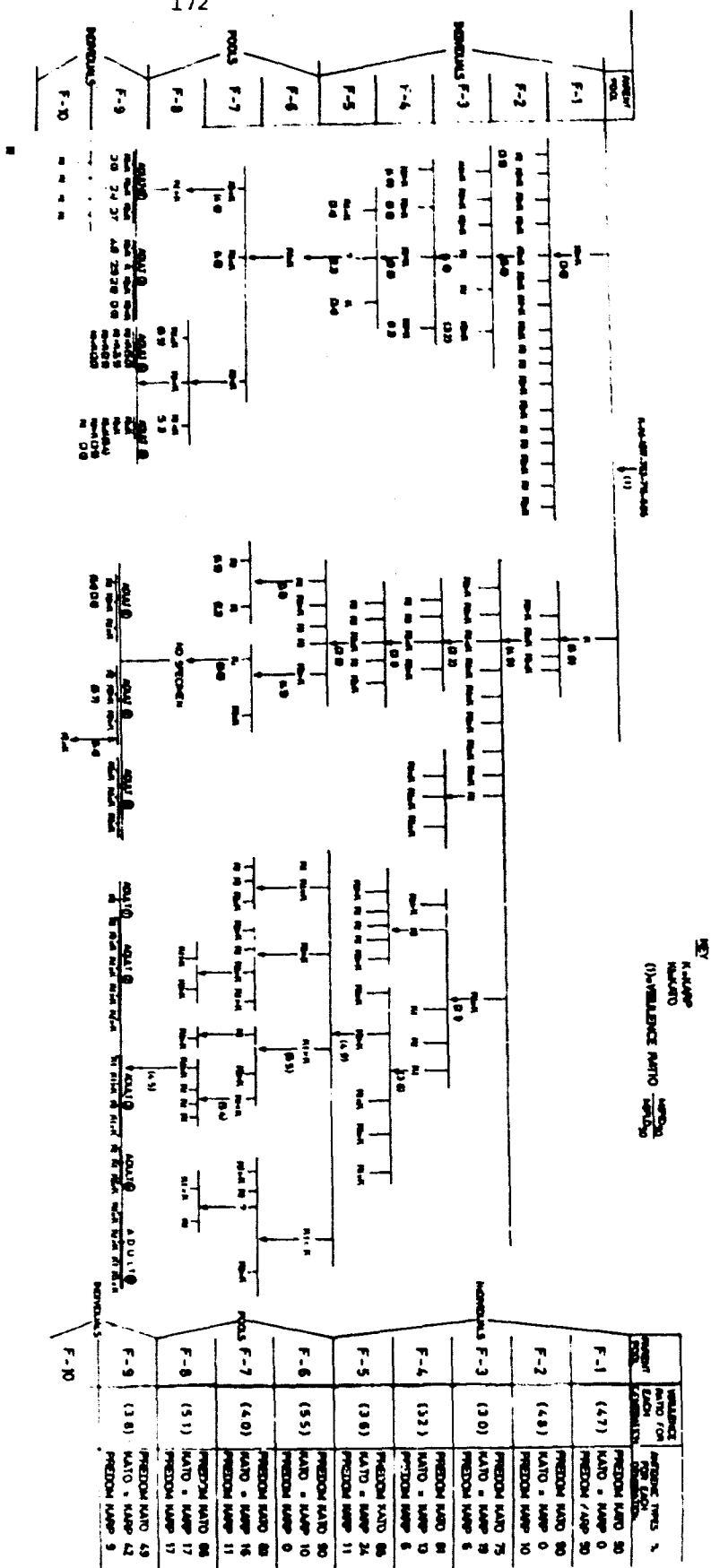
As determine by strain specific FA conjugates used in conjugation with specific blocking sera. Plaque isolations in tissue culture and identification of strains isolated will be performed at a latter date to confirm the above.

Fig. 25 Strains of R. *tsutsugamushi* in the Positive L. *Fletcheri* Colony for 10 Generations*

the antigens of the two infecting strains, Karp and Gilliam. The
strain specific conjugates used to determine the strain(s) isolated
at 3 month intervals were the same as those employed for the colony,
see above. The results of the study, to date, are presented in
Table 14. At no time during the study was any strain other than
Karp or Gilliam detected. Thus indicating that these two strains
are antigenically stable in *R. awandalei*, a wild rat.

Several other interesting findings were brought to light during
this study in addition to the antigenic stability. Positive isolates
were obtained from all eight of the original animals at 3 months,
from 3 at 6 months and from 3 at 9 months proving that a high
percentage of *R. awandalei* remain chronically infected. However,
only 6 of 14 2nd passage animals became infected and to date none of
the 3rd passage animals have become positive by isolation or serology.
The strain isolated depended both on how long the animal had been
infected and the passage level, see Table 14. At 3 months Karp and
Gilliam were both isolated at equal level and frequency. At 6
months, Karp was the predominant strain and at 9 months only Karp has
been isolated. Of the 4 positive second passage animals, 3 were
infected only with Karp while the 4th yielded an isolate containing
both Karp and Gilliam, however, even there the Karp predominated.
The titers obtained depended on chronic infection. The correlation
between strain(s) isolated and the titers to two individual
antigens was almost perfect (Table 14), as was the correlation
between titers and whether an isolate was obtained (one exception at
5 months).

In summary, the Kato strain of *R. tsutsugamushi* contains a
minor Karp component that phenotypically varies in its expression, but
the strain remained Kato based on its major antigenic component. At
no time were other than the 3 infecting strains (Karp, Kato, and
Gilliam) detected, except for the minor Karp component of Kato in any
of the three hosts *(Leptotrombidium fletcheri, Rattus awandalei* and
Presbytis cristatus) after months or years of chronic infection in
that host.

Attempted Infection of Three Species of Vector Mites
(Leptotrombidium) in the Laboratory: These studies were initiated
two years ago and preliminary data were reported last year, see
USAMRU, Malaysia, Annual Report 1971 for background, technique, etc.
All results to date are reported here so that valid comparisons can
be made. The data on uptake of rickettsia from infected rodents
(mice and rats) represented by isolations obtained from two individuals
at each development stage and transovarial transmission are presented
in Tables 15 through 19. It can be seen that larvae of the three
vectors of *Leptotrombidium* mites, *L. (L.) deliense, fletcheri* and
arenicola can take up rickettsial organisms from infected rodents
during feeding on infected rodents, however, to date, no transovarial
transmission has been detected, even into the F-2 larvae in one
experiment.

Table 15

Attempted Infection of Two Species of Leptotrombidium Mites with the Karp Strain of *R. tsutsugamushi*

L. (L.) deliense

Mouse No. & Sensitization	Site of Feeding	Development State and Time After Feeding					No. of Adults	No. of Adults Laying Eggs	No. of F-1 Offspring Individually Tested No. POS/Total	No. of F-2 Pools Tested Composed of 1-10 Larvae No. POS/Total
		Post Larval (24 hrs)	Prenymph (7 days)	Nymph (13 days)	Teliophane (31 days)	Adults (45 days)				
1 B*	ear	-**	+***	ND†	-	-	4	4	0/19	0/1
1 B*	back	-	-	-	-	-	0	0	ND	
2 B	ear	-	-	-	-	-	8	7	0/38	0/12
2 B	back	-	-	-	ND	ND	2	1	0/3	0/2
3 A	ear	-	-	-	-	-	9	5	0/36	0/7
3 A	back	-	-	-	ND	ND	2	2	0/14	0/4
4 A	ear	-	-	-	-	-	7	2	0/16	
4 A	back	-	+	+	ND	ND	4	4	0/24	

L. (L.) fletcheri

Mouse No. & Sensitization	Site of Feeding	Post Larval (24 hrs)	Prenymph (7 days)	Nymph (13 days)	Teliophane (31 days)	Adults (45 days)	No. of Adults	No. of Adults Laying Eggs	No. of F-1 Offspring Individually Tested No. POS/Total	No. of F-2 Pools Tested Composed of 1-10 Larvae No. POS/Total
5 B	ear	-	-	ND	-	-	7	2	0/8	
5 B	back	-	-	ND	ND	ND	0	0		
6 B	ear	-	-	-	ND	ND	5	0		
6 B	back	-	-	ND	-	-	0	0		
7 A	ear	-	-	ND	ND	ND	9	5		
7 A	back	-	-	-	ND	ND	0	0		
8 A	ear	-	-	ND	-	-	3	0		
8 A	back	-	-	-	-	-	6	2		
8 A	back	-	-	-	-	-	2	0	0/19	

1 B* : B = *Blankaartia sp.* sensitized mouse : A = *L. (L.) fletcheri* sensitized mouse.
-** : Negative
+*** : Positive including back-challenge with the Karp strain.
ND† : Not done
Note : Challenge dose of Karp in the mice was $10^{6.7}$ MIPLD$_{50}$'s, route IP

Table 1c

Attempted Infection of Two Species of Leptotrombidium Mites with the Karp and Gilliam Strains of R. tsutsugamushi

a. (L.) deliense

b. (L.) fletcheri

Mouse No. and No. Exp.	Site of Feeding	Demonstrable IF and Time after Feeding								No. of Adults Laying Eggs	No. of F-1 Offspring Individually Tested No. POS/Total No.	No. of F-2 Pools Tested of 4-10 Larvae No. POS/Total No.
		First Instar	Proto- nymph	Deuto- nymph	Adults							
3-A									1	0/5	pending
3-A									0		
5-A									2	0/10	
5-A									3		
5-A									3	0	
5-A									0		
17-A									1	0/6	
17-A									2	0/10	
17-A									1	0/15	
17-A									1	0/5	
1-B									6	0/30	
1-B									1	0/5	
1-B									5	0/22	
1-B									7	0/15	
1-B									5	0/24	
1-B									1	0/5	
1-B									0		
1-B									0		
1-B									2	0/8	
1-B									3	0/15	

Mice . . Karp $10^{6.1}$ MIFID$_{50}$'s, and Gilliam $10^{6.5}$ MIFID$_{50}$'s, route IP

Table 17

Attempted Infection of Two Species of Leptotrombidium Mites with the Gilliam and TA 686 Strains of *R. tsutsugamushi*

Mouse No. and Sensitization	Site of Feeding	Development Stage and Time after Feeding					No. of Adults Laying Eggs	No. of F-1 Offspring Individually Tested No. POS/Total No.
		Post Larval (24 hrs)	Prenymph (7 days)	Nymph (13 days)	Teliophane (31 days)	Adults (45 days)		
L. (L.) deliense								
19 A	ear	-	+	-	-	-	6	0/30
20 A	ear	-	-	-	-	-	6	0/49
21 A	ear	-	-	-	-	-	12	0/9
L. (L.) fletcheri								
22 A	ear	-	-	-	-	-	6	0/30
23 A	ear	-	-	-	-	-	3	0/15

Note: Infecting dose for the mice was Gilliam $10^{3.6}$ MIPID$_{50}$ and TA 686 $10^{3.4}$ MIPID$_{50}$'s, route IP

Table 18

Attempted Infection of Three Species of Leptotrombidium Mites with the Gilliam, TA 686 and a Kato Chigger Isolate of *R. tsutsugamushi*

L. (L.) deliense

Mouse No.	Infecting Strains	Development Stage and Time After Feeding					No. of Adults Laying Eggs	No. of F-1 Offspring Individually Tested No. POS/Total No.
		Post Larval (24 hrs)	Prenymph (7 days)	Nymph (13 days)	Teliophane (31 days)	Adults (45 days)		
24	Kato (colony)	-	-	-	-	-	2	0/9
25	Kato (colony)	-	-	-	-	-	7	0/30
26	Gilliam & TA 686	+	-	ND ND	ND ND	ND	1	0/7
27	Gilliam & TA 686	-	-	-	+	+	2	0/11

L. (L.) fletcheri

28	Kato (colony)	-	-	+	-	-	0	0/7
29	Kato (colony)	-	-	-	-	-	1	0/21
30	Gilliam & TA 686	-	-	-	-	-	5	0/7
31	Gilliam & TA 686	-	+	-	-	-	2	

L. (L.) arenicola

| 32 | Kato (colony) | - | - | - | + | ND | 1 | 0/5 |
| 33 | Gilliam & TA 686 | - | - | - | + | - | 2 | 0/12 |

Note: Infecting doses for the mice were Kato (colony isolate) $10^{4.5}$ MIPID$_{50}$'s, Gilliam $10^{4.2}$ MIPID$_{50}$'s, TA 686 $10^{5.4}$ MIPID$_{50}$'s, route IP.

Table 19

Attempted Infection of Two Species of Leptotrombidium Mites Feeding on Wild Rats (R. *annandalei*) Infected with Two Combinations of Three Strains of R. *tsutsugamushi*.

Rat No.	Infecting Strains	Hours Post-inoculation that the Unengorged Larvae were Placed in the rats' ears	Development Stage and Time after Feeding				
			Post Larval (24 hrs)	Prenymph (7 days)	Nymph (13 days)	Teliophane (31 days)	Adults (45 days)
	L. (L.) deliense						
1	Karp : TC 586	48	-	+	-	-	ND
		96	-	-	-	-	ND
2	Karp & TC 586	48	-	-	-	OT	OT
		96	-	-	-	OT	OT
3	Karp & TC 586	48	-	+	-	-	-
		96	-	-	-	OT	OT
4	TC 586 - Kato (colony)	48	-	-	-	-	-
		96	-	OT	-	-	ND
5	TC 566 - Kato (colony)	48	-	-	-	ND	ND
		96	+	-	-	-	OT
	L. (L.) arenicola						
6	Karp - TC 586	48	-	-	-	OT	OT
		96	-	-	-	OT	OT
7	TC 586 - Kato (colony)	48	-	-	-	OT	OT
		96	ND	-	-	OT	OT
8	TC 586 - Kato (colony)	48	-	-	-	OT	OT
		96	-	-	-	OT	OT

OT* : On test, results not completed to include back-challenge.

Note: 48 and 96 hours are the hours post-inoculation that 20 chiggers were placed in each ear of the rat. The first group (48 hrs) were allowed to drop off and then at 96 hrs the 2nd group were placed on the same animal.

Inoculation: Karp $10^{6.8}$ MIPLD$_{50}$ TC 586 $10^{5.5}$ MIPID$_{50}$, and Kato (colony isolate) $10^{3.6}$ MIPID$_{50}$, route IP.

The efficiency with which larvae take up rickettsia from infected rodents appears to depend on the species of *Leptotrombidium* mite. For example, out of 230 individuals checked at various development stages of *L. (L.) deliense*, 17 were positive (7%) while only 2 out of 164 were positive (1%) in *L. (L.) fletcheri* and 4 out of 53 were positive (8%) in *L. (L.) arenicola*. It appears that whether they feed in the ear or on the back makes no difference, nor does prior "sensitization". Tables 15, 16 and 17 present data on "sensitized" feedings and Tables 18 and 19 on "nonsensitized" feedings. There is no difference in frequency with which larvae take up rickettsia. The strain of *R. tsutsugamushi* appears to make no difference in uptake of rickettsia (tests completed for Karp, Gilliam, TA 686, and a Kato isolate from the 7th generation of the positive *L. (L.) fletcheri* colony), Tables 15 through 18. Nor does strain appear to affect transovarial transmission (strains on which tests have been completed are Karp, Gilliam and TA 686). Except for experiment 4 (Table 18), all positive isolates from individuals were at the post larval and prenymph stages. However, experiment 4 (Table 18) presents data on one positive nymph *(fletcheri)* or 4 positive teliophanes *(arenicola)* and two positive adults *(deliense)*.

In conclusion, it appears that larvae of three vector mites of scrub typhus can obtain rickettsia from infected rodents (frequency depends on species of mite). However, no transovarial transmission has been demonstrated to date. Two species of rodents, 5 strains of *R. tsutsugamushi* including a chigger adapted Kato strain and 3 species of vector mites have been tested in various combinations.

Freezing and Storage of *R. tsutsugamushi* Suspensions: The storage of rickettsial suspensions at high titer has been and remains a problem for periods exceeding six months. *R. tsutsugamushi* and other rickettsial organisms can be recovered from stored material (-60 to -80°C) following years of storage, however, a suspension containing 10^{7+} organisms will lose approximately 1-2 $\log_{10}$ of titer for every six months of storage.

Studies of bacteria, virus and mammalian cell cultures have shown that in most cases -175°C or lower (nitrogen) will significantly decrease storage losses and that freezing and thawing rates influence the recovery rate. Other factors such as the composition of the storage media or suspension also play a major role.

A study was designed to investigate the effect of storage temperature, freezing rates and thawing rates on the survival of the Karp strain of *R. tsutsugamushi* stored as a 20% suspension of mouse liver and spleen in Snyder's diluent:

A group of 40 mice were infected (IP) with a dose of $10^{6.0}$ MIPLD$_{50}$ of the Karp strain and euthanized on day 5. Their spleens and livers were removed and a 20% suspension made in cold (4°C) Snyder's diluent. The first study was an accelerated storage study and involved 4 freeze-thaw cycles every 21 days. At each sampling

time, all ampules (containing 1 ml of the suspensicn) were thawed and refrozen with the exception of the one ampule that was titrated in mice. The ampules for fast freeze were placed directly in liquid nitrogen vapor giving a measured freezing rate of 71°C per minute. Those slow frozen were placed in a -20°C freezer giving a rate of 2°C per minute. After 1 hour the ampules were subdivided and placed at their assigned storage temperature. The two thawing rates were controlled by setting the ampules on the laboratory bench (slow thaw) giving rates of 10°C/min. for -20°C storage, 17°C/min. for -65°C and 25°C/min. for -175°C, and placing them in a circulating water bath at 37°C until thawed (fast thaw). The rates for the fast thaw treatment were 37°C/min. for -20°C storage, 65°C/min. for -65°C and 90°C/min. for -175°C storage.

The results of the accelerated storage study are presented in Table 20. The freezing and thawing rates did not significantly affect the titers even though 4 freeze-thaw cycles. The only significant factors were storage temperature and time (or freeze-thaw cycles). This only occurred at -20°C storage at which the titers dropped significantly with time and number of freeze-thaw cycles. It appears that there might be a slight advantage to storage at -175°C, however, the differences between -60°C and -175°C are less than one log_{10} and are not significant.

At the same time, a long term study was set up using the same treatments. However, only one freeze-thaw was involved and the study was set up to run through one year of storage at the three temperatures, -20°C, -60°C, and -175°C. The results are given in Table 21. As in the accelerated storage study, freezing and thawing rates had no consistent effect on titer. It was known before the study was designed that -20°C would be unsatisfactory but it was included as a negative control and to accentuate any effect that freezing on thawing rates might have. The materials stored at -60°C and -175°C were essentially equal in titer at 3 and 6 months. Both materials at -60°C and -175°C, lost approximately 1 log_{10} titer in 6 months.

Isolations from Small Mammals and Serology: Data reported last year (USAMRU, Malaysia, Annual Report 1971), indicated that arboreal mammals are not involved in the scrub typhus cycle in nature while ground and semiarboreal dwelling mammals were equally involved. Based on these results, a long term (3+ years) study was designed to study all known factors that could influence the prevalence ratios of scrub typhus in small mammals. This is a joint project involving the Department of Rickettsial Diseases, Department of Medical Ecology and Department of Acarology.

The study was started in mid August 1971 and results through January 1972 are reported in Table 22. It appears that there are vast species differences within each habitat, for example, *R. whiteheadii* had very low rates of isolation and serologic positives for both the secondary and primary forest while *Tupaia glis* had isolation frequencies of over 70%. As yet unexplained are the low

Table 20

An Accelerated Storage Study of the Effect of Storage Temperature, Freezing Rate
& Thawing Rate on Titers of Liver & Spleen Suspensions of *R. tsutsugamushi*

Treatment	Prefreezing	Time of Sampling and Number of Freezing and Thawing Cycles				
		1 Cycle (1 day)	2 Cycles (7 days)	3 Cycles (14 days)	4 Cycles (21 days)
Freezing rate					
fast	≥ 7.5*	7.5	7.1	5.6	4.8
slow	7.5	7.5	7.4	5.5	5.5
Thawing rate					
fast	7.4	7.4	7.4	5.9	5.1
slow	≥ 7.5	7.5	7.2	5.3	5.1
Storage temperature					
-20°C	≥ 7.5	7.3	7.0	3.6	1.3
-60°C	≥ 7.5	7.5	7.4	6.4	6.8
-175°C	≥ 7.5	≥ 7.5	7.5	6.8	7.1

≥ 7.5 = Equal to or greater than $10^{-7.5}$ MIPLD$_{50}$'s/0.2 ml of a 20% suspension of
mouse liver and spleen in Snyder's diluent. Each titer shown for each
treatment is the mean titer for that treatment across the other
treatments at the given sampling period.

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Table 21

A Long Term Storage Study of Mouse Liver and Spleen Suspensions of *R. tsutsugamushi*, (Karp Strain); Measuring the Effect of Storage Temperature, Freezing Rate and Thawing Rates

Treatments	Prefreeze	Storage Time						
		1 day	7 days	14 days	21 days	30 days	3 months	6 months
Freezing								
fast	7.7*	7.3	5.2	6.6	5.5	4.2	5.0**	5.8
slow	7.7	7.3	5.3	5.8	4.9	3.6	5.7	5.9
Thawing rate								
fast	7.8	7.3	5.3	5.9	5.5	3.6	5.5	5.6
slow	7.6	7.2	5.1	6.5	4.9	4.1	5.2	6.1
Storage temperature								
-20°C	7.7	7.0	4.9	5.0	2.9	1.0	<1.0	<1.0
-60°C	7.9	7.3	5.7	7.8	6.7	5.9	5.5	5.4
-175°C	7.5	7.5	5.0	6.8	7.2	4.7	5.2	6.3

7.7* = $10^{-7.7}$ MIPLD50's/0.2 ml of 20% suspension of mouse liver and spleen in Snyder's diluent. Each titer shown for each treatment is the mean titer across the other treatments (mean of 6 titers for freezing and thawing rates and 4 for storage temperature).

5.0**= The mean titers for the freezing and thawing rates at 3 months increased over 30 days as the samples stored at -20°C were negative and not included in the calculations.

Table 22

Small Mammal Isolations and Serologic Results from Five Habitats Located
within a Mile Radius

Habitat	Species	Blood Isolation Number	% Positive	Serology Number	(IFAT) % Positive
	Rattus surifer	–	–	59	8
	Others*	–	–	13	23
Village	*Rattus rattus diardii*	4	0	–	–
	Rattus exulans	87	16	106	12
	Rattus argentiventer	39	21	40	35
	Rattus tiomanicus jalorensis	135	21	137	31
	Total	265	19	355	24
	Rattus surifer	–	–	90	4
	Rattus bowersi	–	–	14	43
Lalang Grass	*Rattus exulans*	16	19	17	12
	Rattus tiomanicus jalorensis	151	28	183	47
	Rattus argentiventer	61	34	70	51
	Total	228	29	374	36
	Rattus whiteheadi	–	–	26	0
	Rattus surifer	–	–	130	7
	Rattus rajah pellax	–	–	16	44
Edge Habitat	Others**	–	–	24	29
	Rattus tiomanicus jalorensis	48	27	58	47
	Rattus sabanus	10	30	6	33
	Rattus argentiventer	14	43	22	41
	Total	72	31	282	22
	Rattus tiomanicus jalorensis	2	0	1	0
Relict	*Rattus whiteheadi*	33	9	35	3
Primary/	*Rattus surifer*	40	15	51	18
Secondary	*Rattus rajah pellax*	20	15	28	14
Forest	*Rattus sabanus*	31	32	28	32
	Tupaia glis	12	75	14	43
	Total	138	22	157	18
	Rattus whiteheadi	57	2	95	1
Mostly	*Rattus surifer*	34	18	98	13
Primary	*Rattus sabanus*	73	21	95	36
Forest	Others***	6	33	13	62
	Rattus rajah pellax	29	41	51	29
	Tupaia glis	14	71	18	44
	Total	213	22	371	21

Note: For methods see USAMRU (Malaysia) Annual Report 1971
Others*: Include *Rattus muelleri, Rattus rajah pellax* and *Rattus bowersi*
Others**: Include *Rattus muelleri, Rattus bowersi* and *Tupaia glis*
Others***: Include *Rattus muelleri* and *Rattus tiomanicus jalorensis*

frequencies of serologic positives for the two types of forests in relation to isolation rates and serological data reported last year.

For a complete ecologic description of the study area see the Medical Ecology section of the Annual Report. The Acarology section of this report contains the data on the *Leptotrombidium* mites collected from rodents and black plates.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY							30 06 72	DD-DPA.E(AR)636

30 06 71		U		N/A		NL	☐ YES ☐ NO	△ WORK UNIT

a. PRIMARY		3A062110A831				
b. CONTRIBUTING						
c. CONTRIBUTING						

Investigations of Tick Typhus

010100 Microbiology

10 71	9 72		

DADA17-72-G-9350

a. DATE EFFECTIVE 10 71	EXPIRATION 10 72		RESOURCES ESTIMATE	PROFESSIONAL MAN YRS	FUNDS (in thousands)
b. NUMBER		FISCAL YEAR	72	1.0	21.7
c. TYPE Y Grant	d. AMOUNT 218				
e. KIND OF AWARD	f. CUM. AMT.		73	1.0	23.4

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RESPONSIBLE INDIVIDUAL NAME Dr. R. Bhagwan Singh, Acting Director TELEPHONE Institute for Medical Research	PRINCIPAL INVESTIGATOR NAME Roberts, L.W., CPT, MSC TELEPHONE SOCIAL SECURITY ACCOUNT NUMBER ASSOCIATE INVESTIGATORS NAME: NAME:

Tick typhus, ixodid ticks, rickettsia, hemolymph screening

23.(U) Technical Objective: To make serological and epidemiological studies of tick typhus in West Malaysia.

24.(U) Approach: Ticks will be collected from areas where tick typhus cases have occurred; a hemolymph test will be used to detect rickettsia in ticks.

25.(U) Progress: A total of 105 ixodid ticks of 6 species were collected from an area where tick typhus had previously occurred and were screened for rickettsial infection by examination of hemolymph slides. Examination of both giemsa and indirect fluorescent antibody preparations indicated that 2 *Haemaphysalis papauana nadohatrami* were infectious with a Rocky Mountain Spotted Fever-like rickettsia. *Rickettsia canada*-like infections were indicated by hemolymph screening in 2 *H. p. nadohatrami* and 1 *H. semermis*. These ticks are being colonized for further study.

INVESTIGATIONS OF TICK TYPHUS

General

Tick-borne rickettsioses in West Malaysia have been studied periodically since 1959. A 2 year study during 1962-63 demonstrated a potential for tick-borne disease outbreaks (IMR Annual Report 1964). Serological and epidemiological studies have been made by this Unit on all reported cases occurring in West Malaysia. Although no cases were reported during FY 1972, a study was made of ticks collected in an area where previous cases of tick typhus had occurred.

Methods

Unengorged ixodid ticks were collected from 23-25 March in a disturbed primary dipterocarp forest near Mersing, Johore. Normal tick dragging procedure was found to be ineffective in this area, but adequate collections were made by holding the flannel cloth in front of and against the body and walking slowly through clumps of undergrowth. With this method, 105 ticks were collected and kept alive for screening with the hemolymph technique (Burgdofer 1970). Ticks were maintained singly at 35°C for 48 hours before hemolymph slides were made. To obtain hemolymph, the tarsal segment of leg III was severed. Four spots of hemolymph were placed in a line on one slide for Indirect Fluorescent Antibody preparation: the first was treated with a human Rocky Mountain Spotted Fever conjugate from WRAIR; the second received normal human serum; the third, a *Rickettsia canada* conjugate from a silvered leaf-monkey prepared by the Department of Rickettsiology, and the last spot received normal monkey serum. A giemsa stained hemolymph slide was also made. Suspected positive slides were compared to known positive RMSF and *R. canada* slides. Ticks suspected of being infectious in hemolymph screening were fed individually on guinea pigs for isolation attempts.

Results

The species of ticks collected are shown in Table 1. *Haemaphysalis papauana nadchatrami* and *H. semermis* were the most common species collected. Of the *H. p. nadchatrami*, 2 were indicated to be infectious by IFAT and giemsa examination for Rocky Mountain Spotted Fever-like rickettsia. One was a female that has since oviposited, producing approximately 300 eggs. The guinea pig on which this tick initially fed died 9 days after attachment. The tick reattached on a second guinea pig, and isolation attempts are in progress. Two other *H. p. nadchatrami* appeared to be infectious with RMSF-like rickettsia under IFAT, but no rickettsia were seen on the giemsa slide. No isolations have yet been made from guinea pigs for these ticks. *R. canada*-like infections were indicated by hemolymph screening in 2 *H. p. nadchatrami* and 1 *H. semermis*. Attempts will be made to establish infectious colonies of ticks for further study.

Table 1

Species of Ticks Collected 23-25 March in a Disturbed Dipterocarp
Forest near Mersing, Johore, West Malaysia

Species Collected	Males	Females	Total
Amblyomma festudiinasium	1	-	1
Dermacenter auratus group (close-spur)	5	4	9
Dermacenter auratus group (wide-spur)	2	3	5
Haemaphysalis bispenosa	4	12	16
H. papauana nadohutrami	13	26	39
H. semermis	22	13	35
Total			105

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19. Donaldson, J.R. & Kyser, K.A. The clinical and biological implications of the apparent dynamics of the diarrhea in Malaysia. (Presented at the Seminar of the Malaysian Society of Parasitology & Tropical Medicine held in Kuala Lumpur.)

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21. Muul, I. & Lim, B.L. Behavior and ecology of small mammal populations in a Tropical Forest. (Presented at the Annual Meetings of the American Society of Mammalogists held in Florida State University, Tampa.)

22. Kyser, K.A. The roles of second messengers and antagonist in endotoxin-induced fever and shock. (cleared for presentation)

23. Kyser, K.A. & Cheah, W.C. *In vitro* growth of bacteria-free cultures of *Trichomonas vaginalis*: Advantages of mucolysis of vaginal aspirates, application of taxometry, and maltose-induced growth stimulation. (cleared for presentation)

APPENDIX

List of Investigators

U.S. Army Medical Research Unit

Commanding Officer	-	COL Francis C. Cadigan, Jr., MC
Clinical Pathologist	-	MAJ James R. Donaldson, RAMC
Medical Officer	-	MAJ Kay A. Kyser, MC[1] MAJ Timothy J. Dondero, Jr., MC[2]
Veterinary Officer	-	MAJ Jerry S. Walker, VC
Entomologists	-	CPT Richard G. Andre, MSC CPT Lyman W. Roberts, MSC
Ecologist	-	CPT Illar Muul, MSC
Laboratory Officer	-	Miss Elsie Gan, B.A. (Serologist)
Administrative Officer	-	CPT George A. Kirschbaum, Jr., MSC[3] 1LT Craig R. Lewis, MSC[4]

Malaysian Government Officers

Dr. Lim Teong Wah, Senior Virus Research Officer, IMR.

Mr. Cheong Weng Hooi, Senior Entomologist, IMR.

Mr. Lim Boo Liat, Mammalogist, IMR.

Mr. M. Nadchatram, Acarologist, IMR.

Mr. Yap Loy Fong, Senior Laboratory Assistant, IMR.

Dr. Malcolm Bolton, Senior Medical Officer, Department of
Orang Asli Affairs, Kuala Lumpur.

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1. Departed for USA, April 1972
2. Joined USAMRU January 1972
3. Departed for USA, May 1972
4. Arrived from USA, January 1972

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to anticipating the epidemics in unnatural hosts, such as man, under specific circumstances. For example, the habitat of greatest enzootic activity of scrub typhus *(Rickettsia tsutsugamushi)* in Malaysia is the forest rather than the classical "scrub" habitat previously described. This was found to be the case for areas tested both in West and East Malaysia (Sabah). In both areas arboreal rodents did not appear to be involved, not even as the postulated nidus or primordial source for infection (Audy, 1961, in May, J.M. ed., Studies in Disease Ecology, Hafner, N.Y.). Seasonal effects appear to be operant, although it is not possible to determine at this time whether it is a matter or reinfections or synchronous recrudescence of latent infections. Seasonal effects may obscure the results of short-term surveys. Species seem to respond differentially to infections with zoonotic pathogens as predisposed by their ecological niches. In surveys, trap response has to be considered. In survey trapping, many species of arboreal mammals are missed. These are actually shown to be abundant when other collecting methods (such as capture by hand from arboreal-tree cavities) are employed. Studies with the aid of the canopy transect walkway system (Muul & Lim, 1970, Science, 169: 788-789) have shown that there is little overlap in species diversity in the forest canopy and that on the ground. Rates of parasitization, such as with *Plasmodium* differ also according to vertical zonations. Forests that appear similar differ markedly in their species diversity of mammals depending on the age and history of the forests. Prevalences of various blood parasites also differ in various habitats (e.g. Muul, Lim, and Yap, 1970. S.E. Asian J. Trop. Med. & Publ. Hlth., 1(3): 418-419). Additional data for seasonality studies of ecological phenomena and temporal distribution of zoonotic pathogens are being accumulated and analyzed.

LABORATORY ANIMAL DEVELOPMENT AND ZOONOTIC DISEASES

No major changes were made in the management of the breeding colonies during the last year except for the guinea pigs where new breeding stock was obtained from WRAIR. The rat and hamster colonies were recaged and brought up to standard. A new laboratory animal facility was designed for the IMR in cooperation with the Department of Veterinary Medicine, SEATO Medical Research Laboratory. This will be a modern facility and will bring the laboratory animal facilities at the IMR up to modern standards. Construction is to start 1 November 1972 with a completion date of December 1973.

Procedures were standardized for the capture, handling and conditioning of silvered leaf-monkeys. Current methods are yielding a survival rate of 67%. No losses have been encountered in silvered leaf-monkeys after the first two months in the laboratory and one group has now been in the laboratory over 1 year. In order to cut losses and to obtain a 67% survival rate, it was necessary to take charge of the animals at the time of their capture and immediately bring them to the laboratory. Exhaustion and shock are the largest killers of the animals, most deaths occurring within 5 days of capture.

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Normal values for silvered leaf-monkeys for rectal temperature, PCV, RBC, WBC including differential and serum protein have been determined. It was found that the animals suffer from a normocytic, normochromic anemia in the wild state which corrects itself in the laboratory over an eight week period. Serological work up of monkeys revealed that in contrast to pig-tailed and cynomolgus monkeys the silvered leaf-monkey does not carry antibodies to six of the endemic diseases of Southeast Asia.

Mouse deer survival and breeding continued to improve. The survival rate is now 70% on wild caught animals that arrive at the laboratory within 24 hours of capture. Several offspring were born during the year. The *Rattus annandalei* colony continued to expand and production records revealed a litter size of 4.3 (range 1-7) with a mean interval of 41.5 days between litters.

INVESTIGATIONS ON LIVER *IN VITRO*

Investigations on Normal Liver Tissue *In vitro*

Attempts were made to maintain explants of normal liver *in vitro* in order to study factors which promote liver regeneration and also to attempt to grow the primary exoerythrocytic stage of malarial parasites for possible studies of growth requirements of and drug action on this important stage.

Liver from pig-tailed macaques *(Macaca nemestrina)* was collected at surgery, cut into $1\frac{1}{2}$ mm cubes and placed in collagen lined tubes containing tissue culture medium M 199 and homologous serum and maintained at 39°C. Medium and serum were changed every third day. Biochemical monitors of liver parenchymal functions - production and storage of glycogen, production and excretion of cholesterol and albumin - were adopted in order to evaluate stimulation or inhibition of the liver. At various times of incubation C-14 labelled glucose or sodium acetate was added to the medium and determination was made of the amount of isotope incorporated after 2 days into the tissue glycogen, excreted cholesterol, and albumin. Technical problems were encountered in histological preparation possibly due to the friability of the infarcted cores of the liver pieces.

When isotope labelled substrates were added to the incubation mixtures of explanted liver, M 199 and serum the label was reproducibly incorporated into the three fractions. The times of incubation prior to pulsing with isotope which have been tested thus far are 1 day, 4 days, 8 days, 12 days, 16 days, 20 days, 24 days, 28 days. Longer studies are in progress. Serum collected after partial hepatectomy appeared to promote greater incorporation of isotopes into the three fraction than normal, pre-hepatectomy serum. In general serum collected at 2 and 3 weeks following surgery seemed to stimulate incorporation of label into all fractions. Heat "inactivation" of both pre and post hepatectomy sera at 56°C for 30 minutes caused a

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reduction of isotope incorporation into the three fractions. In preliminary experiments, incorporation of label appeared comparable whether or not collagen was used in the tubes and whether continuous rolling or static conditions were used. Continuous rolling, however, was the routine method for incubation.

Because fairly high background-counts in all three fractions were encountered when heat killed liver was used, and in the chloroform extract and TCA precipitate fractions when no liver tissue was added, further work is under way to confirm and clarify the earlier findings.

INVESTIGATIONS OF MALARIA

Basic investigations on chloroquine resistant *Plasmodium falciparum* and associated mosquito vectors were continued during this reporting period. Studies of malaria in the *Orang Asli* (Aborigines), and the effect of weekly prophylaxis and residual insecticide spraying (DDT) every three months on their malaria rate, was evaluated. Supervision by Gombak Aborigine Hospital staff appears to be the key to the success of the program.

Ecological and entomological research on the mosquito fauna at ground and canopy level have been concluded.

Intensive studies of tragulid (mouse deer) malaria have been carried out. A new species of *Plasmodium* almost three times the size of *P. traguli* has been found. The sporogonic stages of *P. traguli* were studied by light microscopy and the erythrocytic stages by electron microscopy. Transmission experiments were also carried out, with the new species apparently being transmitted by *Anopheles* mosquitoes. Studies of *P. youngi* were initiated. Possibilities exist that the strain is naturally resistant to chloroquine although a secondary exoerythrocytic cycle could be involved. A colony of *A. letifer* has been established and maintained to support the transmission experiments with *P. traguli*, and the new *Plasmodium* species.

Investigations of Malaria *In vitro*

Investigations on the Exoerythrocytic Stages of Malaria *In vitro*

In association with the normal liver tissue culture work attempts were made to reproduce *in vitro* the primary exoerythrocytic stage of *Plasmodium cynomolgi*; as a model for this stage for studies of growth requirements and drug testing.

Salivary glands containing sporozoites of *Plasmodium cynomolgi* were dissected from experimentally infected *Anopheles maculatus*, and added to the liver explants after various periods of culture. At 8, 10, and 12 or 14 days after addition of the sporozoites, liver tissue was inoculated intraperitoneally into a splenectomized pig-tailed macaque *(Macaca nemestrina)*. Simultaneously some of the liver tissues were prepared for histological examination. Medium from the cultures

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was inoculated into a control splenectomized monkey. All monkeys were kept in screened quarters to avoid accidental mosquito borne infection.

Histological preparations of the cultured liver were unsatisfactory, possibly due to the friability of the infarcted cores of the liver.

In the first of the four completed experiments the medium recipient monkey remained negative for patent malarial infection. However, the tissue recipient became positive. The first parasites appeared 32 days after splenectomy, 20 days after the first tissue inoculation, but only 6 days after the last inoculation. This monkey had the appropriate *Plasmodium cynomolgi* with a pattern of parasitemia consistent with a primary infection. This monkey had been in the laboratory for more than four years prior to this work.

In the second experiment the tissue recipient was found to have only three parasites, which appeared 9 days after the last inoculation and 18 days after first tissue inoculation. This was greater than 2 months post splenectomy. While the three parasites were clearly asexual malarial parasites, the species could not be identified with certainty. The medium recipient monkey remained negative.

The subsequent two experiments, which had slight modification of the liver culture technique, did not result in detectable parasitemia.

Further attempts are under way to reproduce the first findings. Further modifications of the histological techniques are being attempted.

Investigations on Drug Resistance of *Plasmodium falciparum In vitro*

Attempts are being made to study the *in vitro* method of Diggs *et al* (*J. Parasit.*: *57*, 187-188, 1971) for field studies in Malaysia. This work is in the preliminary stages. It is anticipated that this method, if feasible, and the Rieckmann method already in use will be employed in the field to determine what correlation might exist between these *in vitro* systems and the parasitological and clinical effect of the drug in the patient.

INVESTIGATIONS OF SCRUB TYPHUS

Microdissection and the direct fluorescent antibody technique were used to demonstrate scrub typhus rickettsia in all stages of infectious *Leptotrombidium (L.) fletcheri (=akamushi)* from the positive colony. The gut tissues and hemolymph were positive in all post egg stages. Unengorged larvae had the highest percentage of tissues positive for rickettsia. Of eggs taken from known infectious females, 91.7% were positive by FA examination. Examination of egg contents with the FA technique appears to be a feasible means for screening field collected vectors for colonization, since the adult is kept alive.

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The infected *L. (L.) fletcheri* colony is into the 11th laboratory generation. Observations of sex ratios in the infectious and non-infectious colonies of this species suggest that a type of parthenogenesis, possibly thelyotokus gynogenesis, is responsible for the lack of males in the positive colony.

L. (L.) deliense numbers are being sampled in a variety of adjacent habitats by black plate and rodent collections. Comparisons are incomplete at present, but monthly fluctuations in numbers do not consistently correspond with average monthly rainfall alone. During some months, marked differences occurred in the average numbers of chiggers on rodents and those on black plates. The number of chiggers on male *Rattus argentiventer* and *R. tiomanicus jalorensis* were notably higher than those on females of the same species. *R. argentiventer* was the most important and *R. exulans* the least important host species in the study area in terms of numbers of chiggers per infested rat.

It was shown in five experiments that the larvae of the vector Leptotrombidium mites *(L. fletcheri, L. deliense, L. arenicola)* can take up rickettsial organisms from infected rodents (mice and rats). However, to date no transovarial transmission has been demonstrated. The efficiency with which vector mites take up rickettsial organisms from infected rodents appears to be species dependent. Unless transovarial transmission occurs in experiments which are not yet completed, it is reasonable to suspect that the vector mites do not become infected from rodents but are themselves both the reservoir and vector of scrub typhus.

Silvered leaf-monkeys seem to be an excellent subhuman primate model for human scrub typhus and all responses measured were strain and dose dependent. Three strains failed to produce eschars at any dose (up to 10^6) while others produced eschars with doses as low as $10^{1.5}$. Significant titers were obtained to the minor antigenic components of the strains in addition to the major components. Complete protection, as determined by clinical illness, was demonstrated in silvered leaf-monkeys challenged at six months with homologous, homologous-heterologous or heterologous combinations of strains. Immunity affected both the formation and duration of eschar formation.

The organism was shown to be antigenically stable in silvered leaf-monkeys, vector mites and a wild rodent. The minor Karp component of Kato varies in its degree of expression both in vector mites and silvered leaf-monkeys.

Storage studies of high titer material showed that within the limits tested freezing and thawing rates had little or no effect on the titers obtained and that through six months storage that there was no significant differences between the titers of the material stored at -65°C and -175°C. Materials stored at both temperature lost 1 $\log_{10}$ of titer in six months.

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An area was selected for a long term study of the effect of season and habitat on mammalian isolation and serology ratios. Indicator species for each habitat were selected and the study initiated for the following habitats: a village area, edge habitat, lalang grass and relict primary/secondary forest.

INVESTIGATIONS OF TICK TYPHUS

A total of 105 ixodid ticks of 6 species were collected from an area where ticks typhus had previously occurred and were screened for rickettsial infection by examination of hemolymph slides. Examination of both giemsa and indirect fluorescent antibody preparations indicated that 2 *Haemaphysalis papauana nadchatrami* . infectious with a Rocky Mountain Spotted Fever-like rickettsia. *Rickettsia canada*-like infections were indicated by hemolymph screening in 2 *H. p. nadchatrami* and 1 *H. semermis*. These ticks are being colonized for further study.